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{Title of Invention} PORPHYRIN COMPOUND CONTAINING BIOTINYL GROUP AND USE THEREOF

{Claims}

{Claim 1} A porphyrin compound containing a biotinyl group represented by Formula (I):

Por-A-Bi

wherein Por represents a porphyrin residue optionally forming a metal complex; Bi represents an optionally substituted biotinyl group; and A represents a C_1-C_{30} hydrocarbyl group, or a C_1-C_{30} heterohydrocarbyl group having 1-10 heteroatoms selected from a group consisting of oxygen, sulfur, and nitrogen.

{Claim 2} The compound according to claim 1, wherein Por is a porphyrin residue that has formed a metal complex selected from a group consisting of heme a, heme b, heme c, variant heme c, heme d, heme d1, siroheme, and heme o.

{Claim 3} The compound according to claim 1 or 2, wherein the Por is a heme b residue.

{Claim 4} The compound according to claim 1, wherein the Por is a porphyrin residue selected from a group consisting of uroporphyrin-I, uroporphyrin-II, coproporphyrin-III, protoporphyrin-IX, and hematoporphyrin-IX.

{Claim 5} The compound according to any of claims 1 to 4, wherein the Bi is a biotinyl group.

{Claim 6} The compound according to any of claims 1 to 5, wherein the A is a straight chain or branched alkylene group of 1-20 carbon atoms, and one or more than one of the non-adjacent CH_2 groups of the alkylene group is optionally substituted by –NH–, –NH–NH–, –NHCO–, –CONH–, –N(C_{1-3} alkyl)–, –O–, –S–, –CO–, –O–CO–, –S–CO–, –O–COO–, –CO–S–, –CO–O–, –CH(halogen)–, –CH(CN)–, –CH=CH–, –NH–NH–CO– or –CO–NH–NH–.

{Claim 7} The compound of any of claims 1 to 6, wherein the A is selected from a group consisting of

–NH–NH–,

–NH–NH–CO–$(CH_2)_n$–NH–,

–NH–NH–CO–$(CH_2)_n$–NH–CO–$(CH_2)_n$–NH–,

–NH–$(CH_2)_n$–NH–,

–NH–NH–CO–$(CH_2)_n$–NH–,

–NH–NH–CO–$(CH_2)_n$–CO–NH–NH–,

$-NH-(CH_2)_n-CO-NH-NH-$, and

$-NH(CH_2)_n-CO-NH-(CH_2)_n-CO-NH-NH-$

in these formulae each n independently represents 1-10.

{Claim 8} A method for preparing the porphyrin compound containing a biotinyl group according to claim 1, comprising reacting a porphyrin optionally forming a metal complex with a compound containing a terminally aminated biotinyl group in the presence of a coupling agent.

{Claim 9} A hemoprotein purification method, comprising a step of performing affinity chromatography using the compound according to claim 1.

{Claim 10} A hemoprotein purification kit, comprising the compound according to claim 1 and carrier beads with an avidin compound bonded thereto.

{Claim 11} A hemoprotein labeling compound that is the compound according to claim 1.

{Claim 12} A method for detecting hemoprotein using the labeling compound according to claim 11.

{Claim 13} A diagnostic agent for hemoprotein-associated diseases, comprising the labeling compound according to claim 11.

{Claim 14} A therapeutic drug for photodynamic therapy, comprising the compound according to claim 4.

{Detail Description}

{0001}

{Technical Field}

The present invention relates to a porphyrin compound containing a biotinyl group, and more particularly, it relates to a porphyrin compound containing a biotinyl group that can purify small amounts of hemoprotein in the living body rapidly and simply. The present invention also relates to a purification method for hemoprotein and apparatus therefor utilizing such a porphyrin compound containing a biotinyl group; a labeling reagent for hemoprotein; a method for the detection of hemoprotein and a diagnostic agent for hemoprotein-associated diseases utilizing that reagent; and a therapeutic drug for photodynamic therapy that contains the above porphyrin compound containing a biotinyl group.

{0002}

{Background Art}

Iron protoporphyrin IX, which is called "protoheme" or simply "heme," performs

various roles as an active center for a plurality of proteins such as an enzyme and oxygen carrier, and also a biosensor (see A. Messerschmidt, R. Huber, T. Poulos, and K. Wieghardt (Eds), Handbook of Metalloproteins Vol. 1, John Wiley & Sons, New York, 2001, etc.). Therefore, detecting and isolating hemoprotein are important for research on these physiological functions.

{0003}

In prior art hemin agarose has been used for the purification of hemoprotein as a carrier in affinity chromatography (Tsutsui & Mueller, Analytical Biochemistry 121, 244-250, 1982: non-patent document 1). However, a problem that cannot be ignored in this prior art method is the non-specific binding between proteins and the agarose that binds to the hemin. Moreover, because of the large particle size of agarose, its protein binding capacity per volume is small, and the spectroscopic detection of its specific binding with the hemoprotein is extremely difficult. In addition, hemin agarose has a shortcoming because it cannot be used for the labeling of hemoprotein.

On the other hand, photodynamic therapy (PDT) for treating diseases such as malignant tumors and rheumatoid arthritis has recently been developed in which a photoactive compound such as a porphyrin is administered to the patient and the treatment site is irradiated with light to activate the porphyrin (Japanese Patent Application Laid-open No. H10-508577: patent document 1). However, therapeutic drugs for PDT that can efficiently supply the photoactive compound to the treatment site have still not been discovered.

{0004}

{Patent Literature 1}

Japanese Patent Application Laid-open No. H10-508577

{Non Patent Literature 1}

Tsutsui & Mueller, Analytical Biochemistry 121, 244-250, 1982

{0005}

{Technical Problem}

Because of these circumstances, it would be desireble if there were provided a hemoprotein purification method that can perform the purification of hemoprotein simply and rapidly. It would also be desireble if there were provided a reagent that can label these proteins to investigate the behavior of hemoproteins (or hemoprotein metabolizing enzymes) in the living body. Further, it would be desireble if there were provided a therapeutic drug for more efficient photodynamic therapy.

{0006}

{Solution of Problem}

The present invention was created to solve the aforementioned prior art problems. The first embodiment of the present invention provides a porphyrin compound containing a biotinyl group represented by Formula (I):

Por-A-Bi

wherein Por represents a porphyrin residue optionally forming a metal complex; Bi represents an optionally substituted biotinyl group; and A represents a C_1-C_{30} hydrocarbyl group, or a C_1-C_{30} heterohydrocarbyl group having 1-10 heteroatoms selected from a group consisting of oxygen, sulfur, and nitrogen. Preferably, the Por is a porphyrin (heme) residue that has formed a metal complex selected from a group consisting of iron-porphyrin derivatives such as heme a, heme b (protoheme IX), heme c, variant heme c, heme d, heme d1, siroheme (Sirohaem), and heme o. More preferably, the Por is a heme b residue. Further, in another preferred embodiment the Por is a porphyrin residue selected from a group consisting of uroporphyrin-I, uroporphyrin-II, coproporphyrin-III, protoporphyrin-IX, and hematoporphyrin-IX. Preferably, the Bi is a biotinyl group.

{0007}

Preferably, in the present invention the A is a straight chain or branched alkylene group of 1-20 carbon atoms, and one or more than one of the non-adjacent CH_2 groups of the alkylene group is optionally substituted by –NH–, –NH–NH–, –NHCO–, –CONH–, –N(C_{1-3} alkyl)–, –O–, –S–, –CO–, –O–CO–, –S–CO–, –O–COO–, –CO–S–, –CO–O–, –CH(halogen)–, –CH(CN)–, –CH=CH–, –NH–NH–CO– or –CO–NH–NH–.

{0008}

More preferably, in the present invention the A is selected from a group consisting of

–NH–NH–,

–NH–NH–CO–$(CH_2)_n$–NH–,

–NH–NH–CO–$(CH_2)_n$–NH–CO–$(CH_2)_n$–NH–,

–NH–$(CH_2)_n$–NH–,

–NH–NH–CO–$(CH_2)_n$–NH–,

–NH–NH–CO–$(CH_2)_n$–CO–NH–NH–,

–NH–$(CH_2)_n$–CO–NH–NH–, and

–NH$(CH_2)_n$–CO–NH–$(CH_2)_n$–CO–NH–NH–

in these formulae each n independently represents 1-10, and preferably 3-7.

{0009}

The second embodiment of the present invention provides a method for preparing the porphyrin compound containing a biotinyl group of Formula (I) above comprising a method for preparing a heme compound containing a biotinyl group that includes reacting a

porphyrin optionally forming a metal complex with a compound containing a terminally aminated biotinyl group in the presence of a coupling agent.

The third embodiment of the present invention provides a hemoprotein purification method comprising a step of performing affinity chromatography using the compound containing a biotinyl group of Formula (I) above.

The fourth embodiment of the present invention provides a hemoprotein purification kit comprising the compound of Formula (1) above and carrier beads with an avidin compound bonded thereto.

The fifth embodiment of the present invention provides a hemoprotein labeling compound wherein a labeling substance is bound to the compound containing a biotinyl group of Formula (I) above.

The sixth embodiment of the present invention provides a method for detecting hemoprotein using the above labeling compound.

The seventh embodiment of the present invention provides a diagnostic agent for hemoprotein-associated diseases comprising the above labeling compound.

Finally, the eighth embodiment of the present invention provides a therapeutic drug for photodynamic therapy comprising a compound wherein Por in Formula (I) is a porphyrin residue.

{0010}

The present invention relates to a compound wherein biotin, which is widely used for labeling and isolating biological polymers because of its high affinity with streptavidin, is bound to heme, which serves as a prosthetic group in many proteins. By using this molecule, the labeling of hemoprotein in the living body, isolation, and purification of small amounts thereof can each be performed rapidly in a single step. Because the porphyrin compound containing a biotin group of the present invention can be bound to various avidin derivatives after it alone binds to the protein, the problems associated with the aforementioned prior art method that uses hemin agarose can be solved.

{0011}

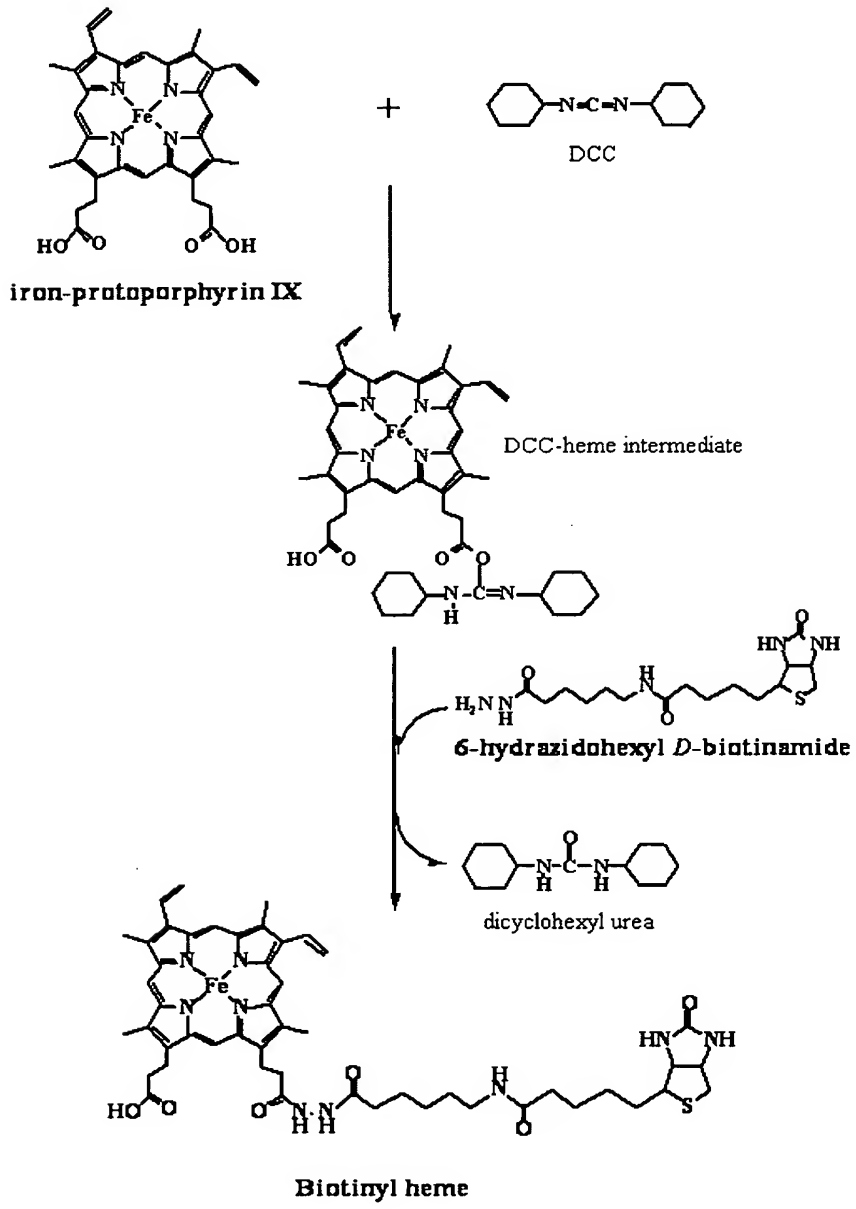
In this description the term "porphyrin" refers to a cyclic tetrapyrrole that is a porphin derivative in which four pyrrole groups linked toghether to form a ring closure by four methine groups; these include, for example, uroporphyrin-I, uroporphyrin-III, coproporphyrin-III, protoporphyrin-IX, and hematoporphyrin-IX, etc. Heme is noted as a most suitable porphyrin that forms a metal complex.

{0012}

In the present description the term "heme" refers to a coordination compound of

porphyrins (or derivative thereof) and mainly bivalent or trivalent iron, and it is also called iron porphyrin and hematin. In the present invention no particular restriction is placed on the heme that is used and a natural heme, for example, heme a, heme b (protoheme IX), heme c, variant heme c, heme d, heme d1, siroheme (Sirohaem), and heme o can be used (see A. Messerschmidt, R. Huber, T. Poulos, and K. Wieghardt (Eds), Handbook of Metalloproteins Vol. 1, John Wiley & Sons, New York, 2001, etc.).

{Chem. 1}



{0013}

In the above formulae, X, Y, and Z each represent the moieties shown in the table below.

	X	Y	Z
Heme b	$-CH=CH_2$	$-CH=CH_2$	$-CH_3$
Heme c	$-C(CH_3)H\text{-}SR^b$	$-C(CH_3)H\text{-}SR^b$	$-CH_3$
Variant heme c	$-CH=CH_2$	$-C(CH_3)H\text{-}SR^b$	$-CH_3$
Heme a	$-CH(OH)-CH_2R'^c$	$-CH=CH_2$	$-CHO$
Heme d	Same as (B)		
Heme d1	Same as (C)		
Siroheme	Same as (D)		
Heme o	$-CH(OH)-CH_2R'^c$	$-CH=CH_2$	$-CH_3$

Note: $SR^b = -CH_2-C(NH-)H-CO-$, $R'^c = -[CH_2CH=C(CH_3)CH_2]_3H$

{0014}

Moreover, in the present invention "heme" is not restricted to the above natural hemes, and various well-known synthetic hemes can be used. For example, such synthetic hemes are described in David Dolphin ed., The Porphyrins, Vol. 1-5, Academic Press, New York, 1978.

{0015}

In the present description the term "hemoprotein" refers to a protein that can bind to a heme such as that noted above (including hemoprotein metabolic enzymes), and it includes, for example, hemoglobin, myoglobin, cytochrome, peroxidase, and catalase, etc.

{0016}

In the present description the term "hydrocarbyl group" refers to an optionally saturated or unsaturated acyclic, or an optionally saturated or unsaturated cyclic, substituted or unsubstituted hydrocarbon, and if the hydrocarbon is acyclic, then it may be either straight chain or branched. Examples of C_1-C_{20} hydrocarbons include, for example, a C_1-C_{20} alkyl group, C_2-C_{20} alkenyl group, C_2-C_{20} alkynyl group, C_1-C_{20} alkoxy group, C_1-C_{20} acyl group, C_4-C_{20} alkyl dienyl group, C_4-C_{20} polyenyl group, C_6-C_{18} aryl group, C_7-C_{20} alkylaryl group, C_7-C_{20} arylalkyl group, C_4-C_{20} cycloalkyl group, C_4-C_{20} cycloalkenyl group, and (C_3-C_{10} cycloalkyl) C_1-C_{10} alkyl group etc. When the hydrocarbyl group is used as an spacer in the present invention, the term refers to a divalent group formed by the removal of one hydrogen atom from one of the aforementioned groups.

{0017}

In the present description the term "alkyl group" refers to an alkyl group that is either straight chain or branched, and includes, for example, a methyl, ethyl, propyl, n-butyl, tert-butyl, pentyl, and hexyl group, etc. Moreover, when the alkyl group is selected as A in the formula, in practice an alkylene group formed by the removal of one hydrogen atom from one of the aforementioned groups can be used as a spacer. Examples of the alkylene group

include a methylene, ethylene, propylene, butylene, pentylene group, and hexylene group, etc.

{0018}

In the present description the term "alkenyl group" refers to a straight chain or branched alkenyl group of 2-20 carbon atoms, preferably 2-10 carbon atoms, having 1 to 3 double bonds; more specifically, it includes ethenyl, 1-propenyl, 2-propenyl, 1-methyl ethenyl, 1-butenyl, 2-butenyl, 3-butenyl, 2-methyl-2-propenyl, 1-pentenyl, 2-pentenyl, 4-pentenyl, 3-methyl-2-butenyl, 1-hexenyl, 2-hexenyl, 1-heptenyl, 2-heptenyl, 1-octenyl, 2-octenyl, 1,3-octadienyl, 2-nonenyl, 1,3-nonadienyl, and 2-decenyl, etc.

{0019}

The term "aryl group" includes, for example, a phenyl group, naphthyl group such as 1-naphthyl and 2-naphthyl, an indenyl group such as 2-indenyl, an anthryl group such as 2-anthryl, a tolyl group such as 2-tolyl, 3-tolyl and 4-tolyl, a biphenyl group, etc.

{0020}

In the present description the term "heterohydrocarbyl group" refers to one of the aforementioned hydrocarbyl groups that also contains at least one heteroatom selected from a group consisting of nitrogen, oxygen, and sulfur, and it includes, for example, a C_1-C_{20} straight chain or branched alkylene group in which one or more than one of the non-adjacent CH_2 groups is optionally substituted by –NH–, –NH–NH–, –NHCO–, –CONH–, –N(C_{1-3} alkyl)–, –O–, –S–, –CO–, –O–CO–, –S–CO–, –O–COO–, –CO–S–, –CO–O–, –CH(halogen)–, –CH(CN), –CH=CH–, –NH–NH–CO– or –CO–NH–NH–.

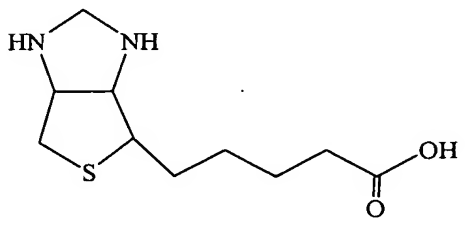
{0021}

Examples of groups that can serve as a substituent of the hydrocarbon group, heterocyclic group, etc., include a halogen atom (for example, fluorine, chlorine, bromide, and iodine, etc.), nitro group, cyano group, optionally halogenated C_{1-6} alkyl group, etc.

{0022}

In the present description, the term "biotinyl group" refers to any residue of biotin shown below, and in a narrow sense refers to a biotin residue shown below from which the hydroxyl group has been removed.

{Chem. 2}



The biotin residue in the present invention may have any substituent provided it does not interfere with the purification, labeling etc., of the hemoprotein. Examples of such a substituent include a halogen atom (for example, fluorine, chlorine, bromide, and iodine, etc.), nitro group, cyano group, optionally halogenated C_{1-6} alkyl group, etc.

{0023}

{Description of Embodiments}

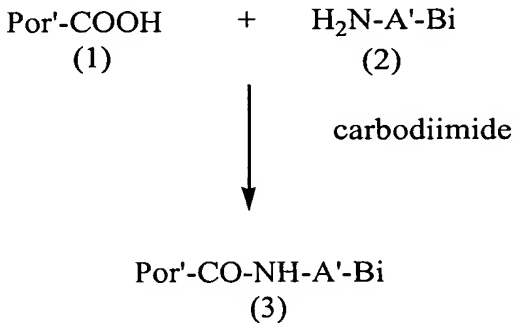
(Preparing method)

The porphyrin compound containing a biotinyl group of the present invention can be synthesized by the method show in scheme (1) below, for example.

{0024}

Scheme (1)

{Chem. 3}



wherein Por' represents a residue wherein one carboxyl group has been removed from a porphyrin optionally forming a metal complex; A' represents a spacer group; and Bi represents a biotinyl group.

{0025}

In scheme (1) above, compound 1 and a terminally aminated biotinyl compound 2 are reacted in the presence of a coupling agent such as a carbodimide, etc., to obtain the target porphyrin compound containing a biotinyl group 3. The terminally aminated biotinyl compound is preferably a hydrazidated biotinyl compound such as biotin hydrazide, 6-hydrazidohexyl-D-biotinamide, 6-(6-hydrazidohexyl) amidohexyl-D-biotinamide (which are well-known compounds on the market), etc. Preferably, this reaction is usually performed in the presence of a suitable solvent at 0°C-100°C, preferably 10°C-40°C, and for 0.5-48 hours and preferably 1-24 hours.

Herein, when a metal complex (for example, a heme compound containing a biotinyl group) is obtained as a final product, the metal complex of the porphyrin (for example, heme) can be reacted with the terminally aminated biotinyl compound in the presence of a coupling

agent, or the porphyrin and the terminally aminated biotinyl compound may first be reacted in the presence of a coupling agent, followed by reaction with a metal (ion) to form the metal complex.

In scheme (I), the porphyrin 1 was illustrated as a model having one carboxyl group, but in practice a porphyrin may have a plurality of carboxyl groups. For example, the target heme compound of the present invention is preferably one in which a single biotinyl group is bonded to a heme. Therefore, it is necessary to adjust the amount of starting material used according to the number of carboxyl groups that the heme in question has. For example, iron protoporphyrin IX has two carboxyl groups. Therefore, if iron protoporphyrin IX is used as porphyrin 1, when 2 or more equivalents, and preferably 2.5 or more equivalents, of porphyrin 1 are used with respect to the hydrazidated biotinyl compound 2, a compound is obtained wherein the biotinylated compound is bonded to only one carboxyl group of the iron protoporphyrin IX via the hydrazide group.

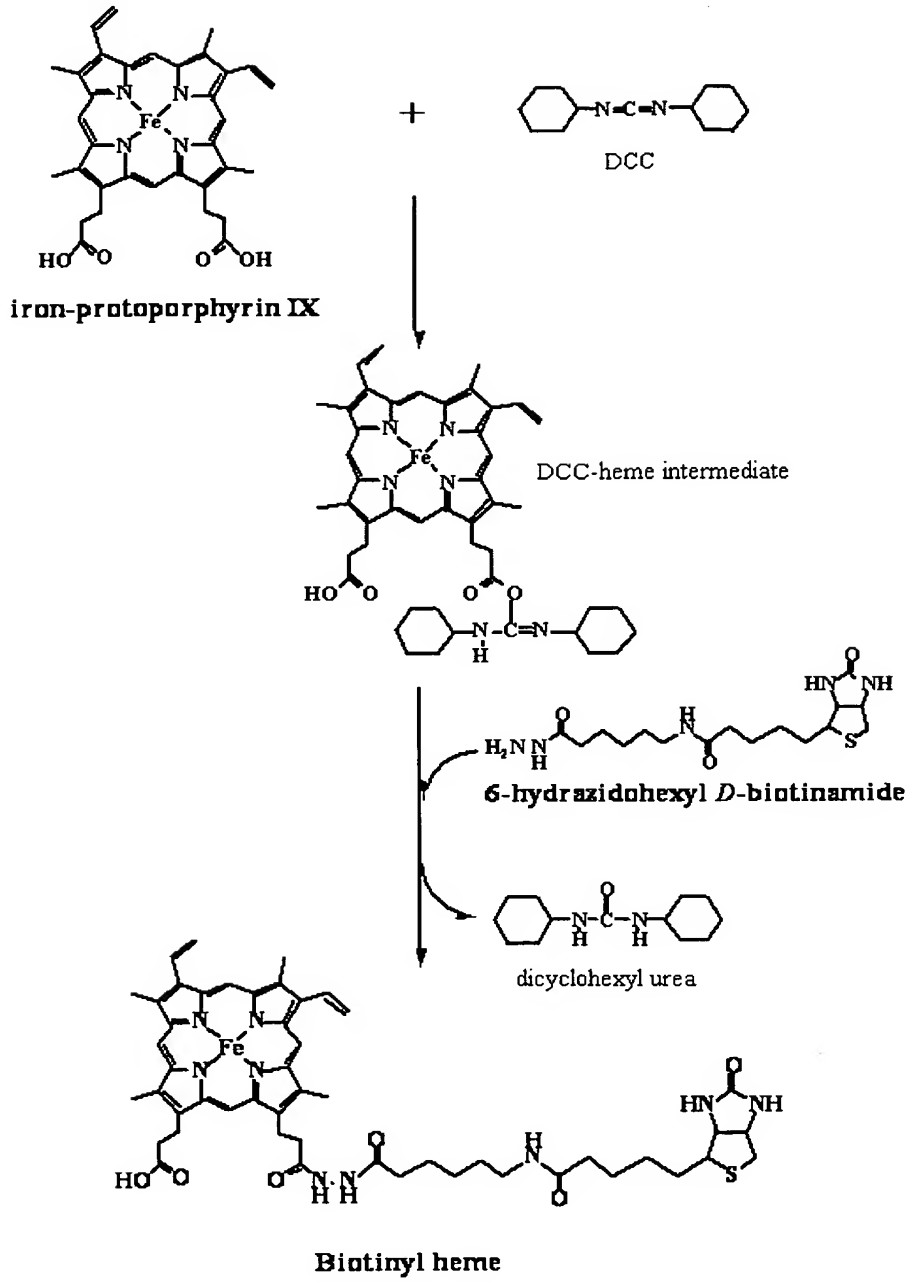
{0026}

For reference purposes to explain the mechanism of the above coupling reaction, scheme (2) shows an example wherein iron protoporphyrin IX and 6-hydrazidohexyl-D-biotinamide are reacted in the presence of a dicarboximide to obtain a porphyrin compound containing a biotinyl group.

{0027}

Scheme (2)

{Chem. 4}



{0028}

Examples of the coupling agent used in this reaction include the following: N,N'-dicyclohexyl carbodiimide (DCC), N'-(3-dimethylaminopropyl)-N-ethyl carbodiimide (DIC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, N-allyl-N'-(β-hydroxyethyl) carbodiimide, N-(α-dimethylaminopropyl)-N'-(β-bromo allyl) carbodiimide, 1-(3-dimethylaminopropyl)-3-(6-benzoyl aminohexyl) carbodiimide, cyclohexyl-β-(N-methyl morpholino) ethyl

carbodiimide, ethyl-1,2-dihydro-2-ethoxy-1-quinolinecarboxylate (EEDQ), isobutyl-1,2-dihydro-2-isobutoxy-1-quinolinecarboxylate (IIDQ), 1-benzotriazolyloxy tris (dimethylamino)-phosphonium hexafluoro phosphate (HBTU), O-{[cyano-(ethoxy carbonyl)-methylidene]-amino}-1,1,3,3-tetramethyl uronium tetrafluoroborate (TOTU), propane phosphonic acid anhydride (PPA), 3-dimethylamino phosphinothioyl-2 (3H)-oxazolone (MPTO), etc.

{0029}

The suitable solvent used in this reaction is not restricted provided the reaction proceeds, and it includes the following examples: aromatic amines such as pyridine, lutidine, and quinoline; halogenated hydrocarbons such as dichloromethane, chloroform, 1,2-dichloroethane, and carbon tetrachloride; aliphatic hydrocarbons such as hexane, pentane, and cyclohexane; aromatic hydrocarbons such as benzene, toluene, xylene, and chlorobenzene; ethers such as diethyl ether, diisopropyl ether, diphenyl ether, tetrahydrofuran, dioxane, and 1,2-dimethoxyethane; amides such as N,N-dimethylformamide and N,N-dimethylacetamide; as well as mixtures of two or more of the above solvents. Especially preferred solvents in the above reaction are dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), or a mixture thereof.

{0030}

If a "base" is used in the aforementioned reaction, it may be selected from the following examples: a basic salt such as sodium carbonate, potassium carbonate, and cesium carbonate; an inorganic base such as sodium hydroxide and potassium hydroxide; an aromatic amine such as pyridine and lutidine; a tertiary amine such as triethyl amine, tripropyl amine, tributyl amine, cyclohexyl dimethylamine, 4-dimethyl aminopyridine, N,N-dimethyl aniline, N-methyl piperidine, N-methyl pyrrolidine, and N-methyl morpholine; an alkali metal hydride such as sodium hydride and potassium hydride; a metal amide such as sodium amide, lithium diisopropyl amide, and lithium hexamethyl disilazide; and a metal alkoxide such as sodium methoxide, sodium ethoxide, and potassium tert-butoxide.

{0031}

Isolation and purification of the end product obtained by the above reaction from the reaction mixture can be performed according to well-known means such as concentration, solvent extraction, fractional distillation, crystallization, recrystallization, and chromatography, etc.

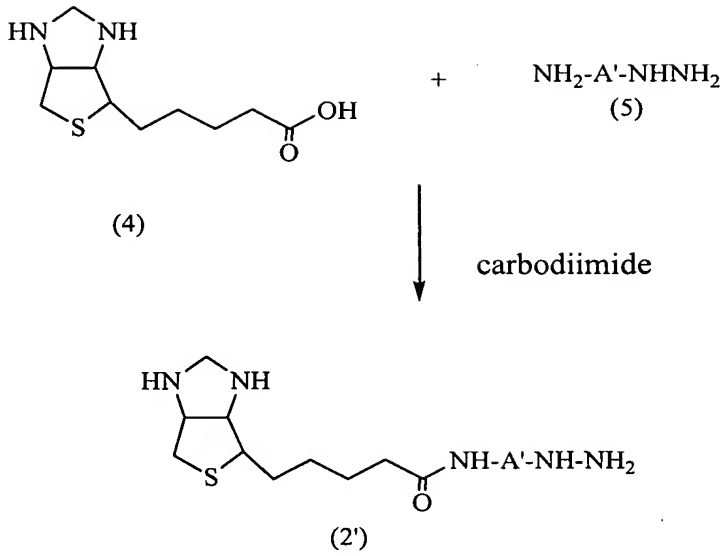
{0032}

For example, the above compound containing a terminally aminated biotinyl group 2 can be synthesized by the reaction shown in scheme (3) below.

{0033}

Scheme 3

{Chem. 5}



{0034}

In scheme (3) above, the terminally aminated biotin compound 2' can be obtained by reacting biotin 4 and a hydrazide compound 5 in the presence of a coupling agent such as a carbodiimide, etc. In place of the hydrazide compound 5, it is possible to use a dihydrazide compound represented by the formula NH_2–NH–CO–A'–CO–NH–NH_2 or a diamine compound represented by the formula NH_2–A'–NH_2 (wherein A' represents a C_1-C_{30} hydrocarbyl group or a C_1-C_{30} heterocarbyl group having 1-6 heteroatoms selected from a group consisting of oxygen, sulfur and nitrogen). These reactions can be performed under the same reaction conditions as the coupling reaction of scheme (1).

{0035}

In this manner it is possible to synthesize a compound in which both ends of the spacer group A in the formula Por-A-Bi are amino groups. Compounds in which the spacer group A is another group can be synthesized by ordinary persons skilled in the art using publicly known organic synthesis methods (see Bayer et al., Methods Biochem. Anal. 26 (1980), 1-45).

{0036}

(hemoprotein purification method)

The purification of the hemoprotein present in the samples is performed by using affinity chromatography with the above porphyrin compound containing a biotinyl group. As

used herein, the term "affinity chromatography" refers to a method for isolating or purifying a target substance contained in a sample (for example, a body fluid sample such as serum, plasma, etc., or a culture supernatant, supernatant obtained by centrifugation, etc.) by utilizing the interactions (affinity) between specific substances such as antigen-antibody, enzyme-substrate, and ligand-receptor interactions. In the purification method of the present invention, isolation or purification of a heme-binding protein contained in the sample is performed by utilizing the specific affinity between the above porphyrin compound containing a biotinyl group and hemoprotein and the specific affinity of the biotinyl group and an avidin compound. The porphyrin compound of the present invention can purify hemoprotein present in a sample in a variety of embodiments using publicly known affinity chromatography techniques. For example, hemoprotein can be purified by a hemoprotein purification kit that contains the porphyrin compound of the present invention and carrier beads with an avidin compound bonded thereto.

{0037}

In accordance with a preferred embodiment of the present invention, first the above porphyrin compound containing a biotinyl group is added to a sample containing the target hemoprotein, enabling the porphyrin compound to bind to the target hemoprotein. Next, an entity wherein an avidin compound such as avidin, etc., is bound to a carrier such as beads, etc., (hereinafter called "avidin beads") is added to the compound wherein the target hemoprotein is bound to the porphyrin compound (hereinafter called the "hemoprotein-porphyrin complex"), and utilizing avidin-biotin binding, the hemoprotein-porphyrin complex is bound to the avidin beads. Thus, the hemoprotein-porphyrin complex that is bound to the avidin beads can be recovered by publicly known means, and the target protein can be isolated and recovered by preparing a suspension in a solution containing a compound having the action of separating heme from protein such as imidazole, acid, guanidine hydrochloride or another denaturing agent, etc. The present invention places no particular restriction on the above carrier provided it is a carrier to which an avidin compound bonded thereto. For example, streptavidin magnetic beads, streptavidin agarose, etc., which are commercially available from Vector Laboratories and Pierce Biotechnology Inc. can be used. Moreover, using magnetized beads has the advantage that the collection can be performed more easily by magnet.

{0038}

(Hemoprotein labeling compound and applications thereof)

The porphyrin compound containing a biotinyl group that was obtained in the above manner can be used as a labeling compound for hemoprotein either alone or by bonding a

labeling substance thereto. In this description, the term "labeling substance" means a substance used to facilitate detection of the presence thereofby physically or chemically bonding to the porphyrin compound containing a biotinyl group. More specifically, this term includes fluorescent substances such as fluorescein isothiocyanate, phycobiliprotein, a rare earth metal chelate, dansyl chloride or tetramethylrhodamine isothiocyanate bonded to an avidin compound such as avidin, streptavidin, etc.; or a radioactive isotope such as ^{3}H, ^{14}C, ^{125}I or ^{131}I, etc. Among these possibilities, avidin compounds are most convenient because they are easy to obtain, and they can simply label the porphyrin compound containing a biotinyl group by utilizing avidin-biotin specific binding.

Hemoprotein contained in a sample (for example, a body fluid sample such as serum, plasma, etc., a culture supernatant or supernatant obtained by centrifugation) can be detected and quantified by publicly known technology using this kind of labeling compound or a diagnostic agent containing this compound. Moreover, the *in vivo* behavior, etc., of hemoprotein can be observed by using such a labeling compound. The diagnostic agent can be prepared in the form of a solution wherein the above compound is stably stored. It can be diagnosed whether a patient has a disease in which a specific hemoprotein is involved by comparing the detected amount of that specific hemoprotein present in the sample with the range of normal values. Heme oxygenase deficiency and leukemia involving a heme-associated transcription factor called Bach are known as such hemoprotein associated diseases. Moreover, the detection of human hemoglobin in feces caused by bleeding of the gastrointestinal organs (occult blood in stool) has been widely used as a method of testing for diseases of the digestive system such as colon cancer in recent years, and the diagnostic method of the present invention can be used to diagnose such a colon cancer.

{0039}

(Therapeutic drug for photodynamic therapy (PDT))

The porphyrin compound containing a biotinyl group of the present invention can be used as a therapeutic drug for PDT. When this porphyrin compound (active ingredient) is used as a therapeutic drug for PDT, it is mixed with a pharmacologically acceptable carrier, excipient, and diluent, etc., and usually administered in the form of an injection.

The active ingredient in the pharmaceutical preparation will be included, for example, in 0.1 to 30 wt%, preferably 1 to 5 wt%. The dose of this therapeutic drug will differ depending on the symptoms, age, weight, etc., of the patient but, for example, the daily amount of the active ingredient administered should be 0.05 mg to 30 mg, preferably 0.05 mg to 5 mg, and more preferably 0.05 mg to 1 mg per 1 kg of body weight of the patient. The content of the active ingredient and the dose are not limited to the above range, and are to be

properly adjusted according to the type of active ingredient, carrier, excipient, diluent, etc., to be used. When PDT treatment is performed, preferably the diseased tissue where the tumor exists will be labeled with avidin before administration of treatment. Such avidin labeling can be performed by using an antibody to the protein (a tumor marker, etc.) expressed specifically by the tumor cells. Then the injection containing the above active ingredient is administered to the diseased tissue. Thus, because of the high affinity between biotin and avidin, the necessary porphyrin compound can be efficiently delivered in a site specific manner to the diseased tissue. Subsequently, the diseased tissue is irradiated with light, and the lesion can be destroyed by activation of the porphyrin. The irradiating light has a suitable wave-length (for example, 600-790 nm) and intensity (for example, 1-50 J/cm^2) for activating the porphyrin. The irradiation of light is performed, for example for 1 minute to 2 hours, preferably 10 to 600 minutes. If necessary, the irradiation of light can be performed by using an optical fiber, etc., inserted in a catheter.

{0040}

{Examples}

The present invention is explained more specifically below based on examples.

{0041}

Example 1: Synthesis of porphyrin compound containing a biotinyl group

Iron protoporphyrin IX chloride (hemin) used as an experimental material was purchased from Sigma. The 6-hydrazidohexyl-D-biotinamide used was purchased from Vector Laboratories.

First of all, hemin and 6-hydrazidohexyl-D-biotinamide were dissolved in dehydrate DMF and DMSO at 6.7 mM and 2.7 mM, respectively. 20 μL of the biotin hydrazide solution and 5.6 mg of dicyclohexyl carbodiimide (DCC) were added to 1 mL of hemin solution. The reaction mixture was gently shaken and incubated in the dark at room temperature for 3 hours. In order to conjugate only one of the two propionate groups of protoheme with the biotin hydrazide, approximately 2.5 equivalent excess amounts of hemin were used for the reaction.

{0042}

The reaction mixture made as above was supplement with approximately 5% (v/v) pyridine and was applied onto a C_{18} reverse-phase preparative HPLC column COSMOSIL $5C_{18}$-ARII (Nacalai Tesque). The porphyrin compound containing a biotinyl group (hereafter, referred to as "biotinyl heme") was eluted with a gradient of 40-60% acetonitrile in the presence of 0.1% TFA. The peak fraction containing the biotinyl heme was collected and immediately lyophilized in the dark. The sample was dissolved in the minimal volume of DMSO and stored at -80°C. The purity of the sample was examined using C_{18} reverse-phase

analytical HPLC column (COSMOSIL $5C_{18}$-AR300, Nacalai Tesque). Identity of the purified molecule was verified by laser-desorption mass spectrometry (MALDI/TOFMS). Figure 1 shows the result of mass spectrometry.

{0043}

This analysis indicated that the obtained compound had the mass of around 969.4 Da. This corresponds to the calculated mass of the biotinyl heme (969.98Da) in which one of the two propionate groups of the protoheme was conjugated with the biotin hydrazide. Therefore, the obtained compound was confirmed to be the biotinyl heme, which is the final compound shown in scheme (2) above.

{0044}

Example 2: Purification of heme protein using the biotinyl heme

Artificial genes encoding sperm whale myoglobin (Springer et al., Proc. Natl. Acad. Sci. USA 84 (1987), 8961-8965), designed globin-1 (DG1) (Isogai et al., Biochemistry 39 (2999), 5683-5690), and designed four-helix bundle hemoprotein (dA1) were cloned into a pRSET-C vector (Invitrogen). The amino acid sequence of dA1, (SEQ ID NO: 1, ML· KKLREEA · LKLLEEF · KKLLEEH · LKWLEGGGGGGGELLKL · HEELLKK · FEELLKL·AEERLKK·L) was designed to form a four-helix bundle in the dimer and to bind one heme per monomer via bis-histidine ligation between the two helices according to the method of Gibney et al. [(Gibney et al., Biochemistry 37 (1988), 4635-4643). A synthetic gene encoding sperm whale myoglobin cloned into pUC19 vector (Springer et al., Proc. Natl. Acad. Sci. USA 84 (1987), 8961-8965) was also used to obtain a cell extract containing the native myoglobin. These hemoprotein-coding vectors were transformed into *E. coli* strain BL21 (DE3). For expression, Terrific Broth (liquid culture medium) supplemented with 100 mg/L ampicillin was grown under control of a T7 promoter using IPTG. Cells were harvested by centrifugation and were washed with 10 mM TRIS-HCl, pH 8.0 and 1 mM EDTA. The resultant pellets were suspended in a lysis buffer containing 6 M urea, 0.5 M NaCl, 1 mM EDTA and 0.1% ODP (octyl glucopyranoside) and were lysed by sonication. After removal of the insoluble fraction by centrifugation, the supernatant was collected and dialyzed with TN buffer. During these procedures, almost all heme associated with proteins in the cell extracts was removed and the proteins were refolded. After the insoluble fraction was removed by centrifugation, those proteins were concentrated to a suitable concentration using Centriprep-10 (Amicon). A cell extract obtained in the above manner was used as a starting material for the purification of a recombinant apohemoprotein using a biotinyl heme.

{0045}

The biotinyl heme was added to the cell extracts obtained as mentioned above in

small increments to finally 10 to 40 μM, and was incubated at 4°C for more than 30 minutes.

Figure 2 shows the changes in the UV-Vis absorption spectra when biotinyl heme was added to the cell extract containing the artificial hemoprotein dA1. In Figure 2, the lowest spectrum shows absorbance without the addition of biotinyl heme, and it is clear that the heme-bound dA1 concentration increases from bottom to top with the stepwise addition of biotinyl heme. The vertical axis shows the absorbance.

{0046}

Next, after the removal of insoluble materials by centrifugation, the solutions were transferred to a sample tube containing streptavidin agarose (Sigma) or streptavidin magnetic beads (Pierce) pre-washed with a washing buffer containing 20 mM TRIS-HCl (pH 8.0), 500 mM NaCl, and 0.5% (v/v) Tween 20. The resultant protein-biotin-heme-streptavidin complexes were collected by centrifugation for the agarose complex or by using magnet for the magnetic bead complex. The pellets were washed twice with the washing buffer and incubated with 10 M imidazol (pH 8.0) to elute the bound proteins. The solution was desalted and lyophilized after removal of the agarose or magnetic beads. The lyophilized samples were dissolved in a small amount of TN buffer and were analyzed by SDS-PAGE with 15% (w/v) polyacrylamide gel. Figure 3 shows the SDS-PAGE electropherogram of the hemoprotein purified by the biotinyl heme. In Figure 3, lane 1 is the molecular size marker (from the top, 94, 67, 43, 30, 20.1, and 14.4 kDa); lanes 2 and 3 are the cell extract and the purified fraction of the recombinant myoglobin, respectively; lanes 4 and 5 are the cell extract and the purified fraction of dA1, respectively; and lanes 6 and 7 are the cell extract and the purified fraction of DG1, respectively.

As shown above, we have prepared three samples containing sperm whale myoglobin, designed globin-1 (DG1), and the designed four-helix bundle heme protein (dA1). Addition of the biotinyl heme into the cell extracts induced the intense Soret absorption bands characteristic of the bound heme in these proteins, indicating that it was effectively incorporated into the protein even in the dense mixture of biological molecules. From these mixtures, the reconstituted hemoproteins were easily collected by streptavidin magnetic beads without significant contamination of other proteins (see Fig. 3).

{0047}

Comparative example 1

After washing the magnetic beads in buffer, instead of adding the imidazole and eluting the apohemoprotein, the same protein was eluted by a process wherein an acid or a denaturing agent such as guanidine hydrochloride was added. In that instance, however, denatured streptavidin subunits that do not bind to the biotinyl heme nor to the beads coeluted

with the hemoprotein. The protein was also purified using streptavidin agarose. However, the use of the agarose increased the contamination due to non-specific interactions of protein with agarose.

In conclusion, it is clear that the biotinyl heme is a useful reagent to detection and purification of native and artificial heme protein. As described above, preparation of the biotinyl heme is simple and its specific ligation with native and artificial hemoproteins can be easily monitored with UV-Vis absorption spectroscopy.

{0048}

Example 3: Bonding of biotinyl heme to myoglobin(labeling)

Apomyoglobin was prepared from horse heart metmyoglobin using the methyl ethyl ketone extraction method described by Ascoli et al. (F. Ascoli, M.R. Fanelli, E. Antonini, Preparation and properties of apohemoglobin and reconstituted hemoglobins, Methods Enzymol. 76 (1981), 72-87). The heme-removed apoprotein was dialyzed against TN buffer containing 10 mM TRIS-HCl (pH 8.0) and 200 mM NaCl at 4°C. After removal of the insoluble fraction by centrifugation, the supernatant was concentrated to 1 to 2 mM with Centriprep 10 (Amicon). Reconstitution of myoglobin with the biotinyl heme was performed by addition of the biotin-heme solution into the apomyoglobin solution in increments of 0.1 to 0.2 equivalents to a small excess of the protein. This mixture was incubated for more than 30 minutes at 4°C and was centrifuged at 20,000xg for 30 minutes. The reconstituted biotin-heme myoglobin was collected in the supernatant and preserved significant stability similar to that of natural metmyoglobin as judged by measurements of the UV-Vis absorption spectrum.

{0049}

The reconstituted myoglobin with the biotinyl heme was diluted with TN buffer to 10 to 20 μM and the UV-Vis absorption spectra were recorded with a Hitachi U-3000 spectrophotometer using a quartz cuvette of 1.0 cm in path length. The ferric, ferrous deoxy and ferrous CO-bond forms were prepared for the spectroscopic measurements according to the above reference by Ascoli et al.

Figure 4 shows the UV-Vis absorption spectra of myoglobin reconstituted with the biotinyl heme with the ferric (solid line), ferrous deoxy (broken line) and ferrous CO-bond forms (dotted line). These spectra were indistinguishable from those of native myoglobin. In addition, it was confirmed that the biotin-hem bound myoglobin preserved stable O_2-binding ability. These results suggest that the biotinyl heme is incorporated in the heme pocket of myoglobin in the manner similar to normal protoheme in myoglobin.

{0050}

{Effects of Invention}

As described above, according to the present invention, the present invention can provide a heme compound containing a biotinyl group that enables the rapid and simple purification of small amounts of hemoprotein in the living body. In addition, the present invention can provide a method for the simple purification of hemoprotein using this kind of heme compound containing a biotinyl group. Furthermore, the present invention can provide a hemoprotein labeling reagent and a diagnostic agent for hemoprotein associated diseases that uses that reagent. Moreover, the present invention can provide a novel therapeutic drug for use with photodynamic therapy.

{0051}

{Sequence Listing}

SEQUENCE LISTING

<110> RIKEN

<120> Biothinyl group-containing porphyrin compound and its use

<130> P03-0029

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Gly Gly Gly Gly Glu Leu Leu Lys Leu His Glu Glu Leu Leu Lys Lys
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Phe Glu Glu Leu Leu Lys Leu Ala Glu Glu Arg Leu Lys Lys Leu
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{Brief Description of Drawings}

{Fig. 1} Figure 1 shows the results of mass spectrography of the heme compound containing a biotinyl group that was obtained in Example 1;

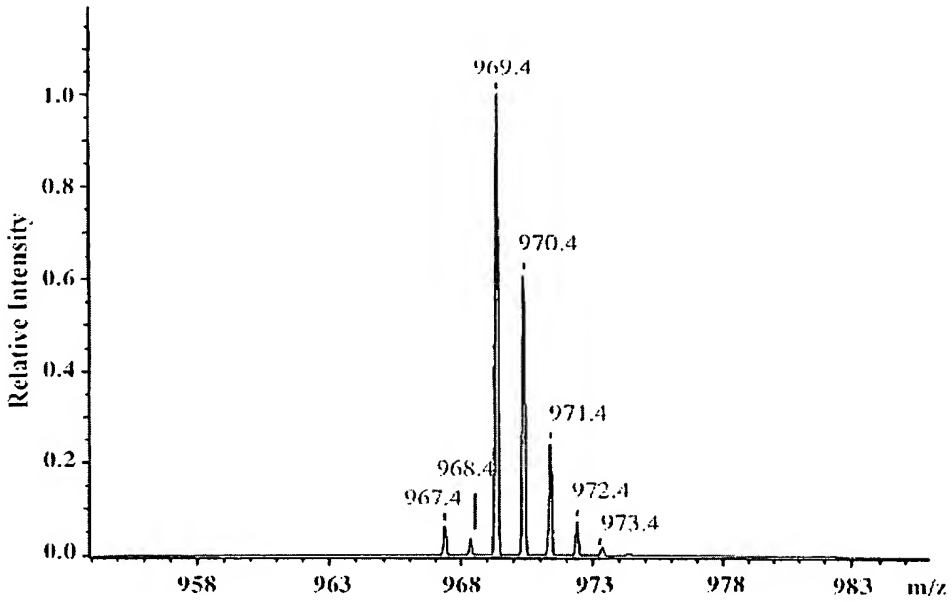
{Fig. 2} Figure 2 shows the change in the ultraviolet-visible light (UV-Vis) absorption spectrum when the biotinyl heme was added to a cell extract containing the artificial heme protein dA1 in Example 2;

{Fig. 3} Figure 3 shows the electropherogram by SDS-PAGE of hemoprotein purified by the biotinyl heme in Example 2; and

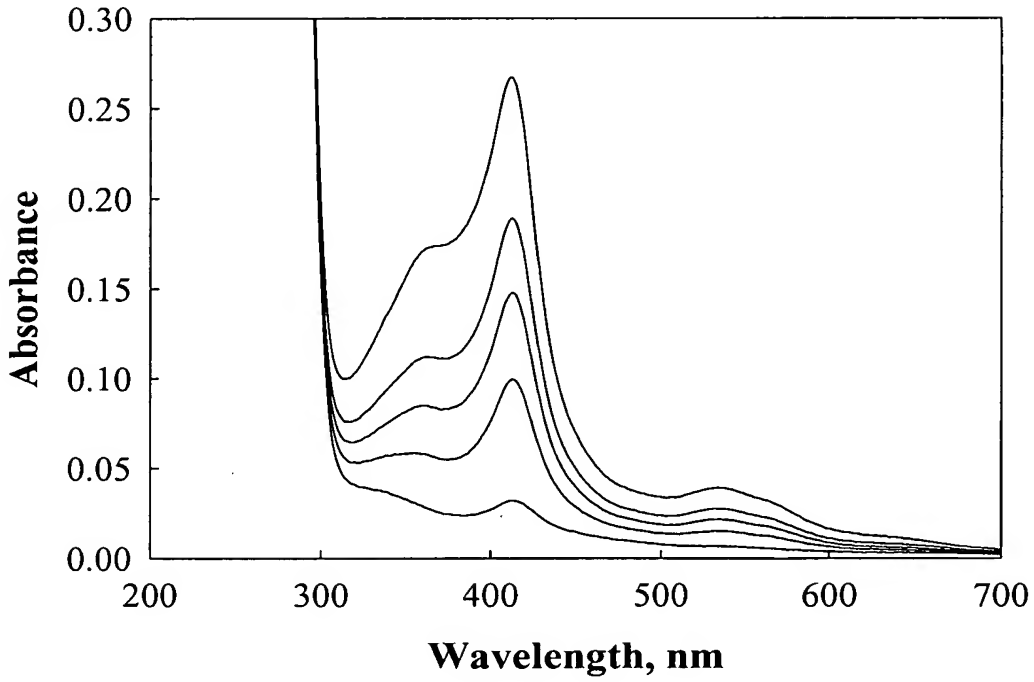
{Fig. 4} Figure 4 shows UV-Vis absorption spectrum of myoglobin reconstituted with the biotinyl heme in Example 3.

{Document name} Drawings

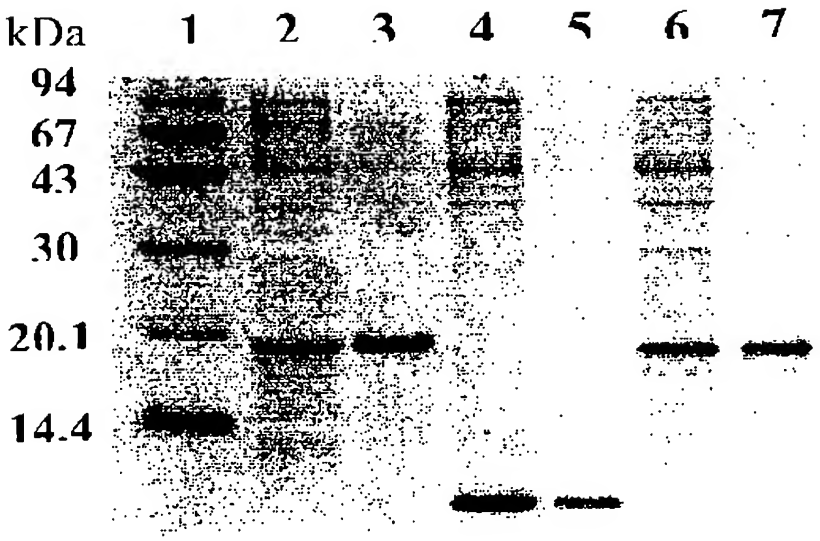
{Fig. 1}



{Fig. 2}



{Fig. 3}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67
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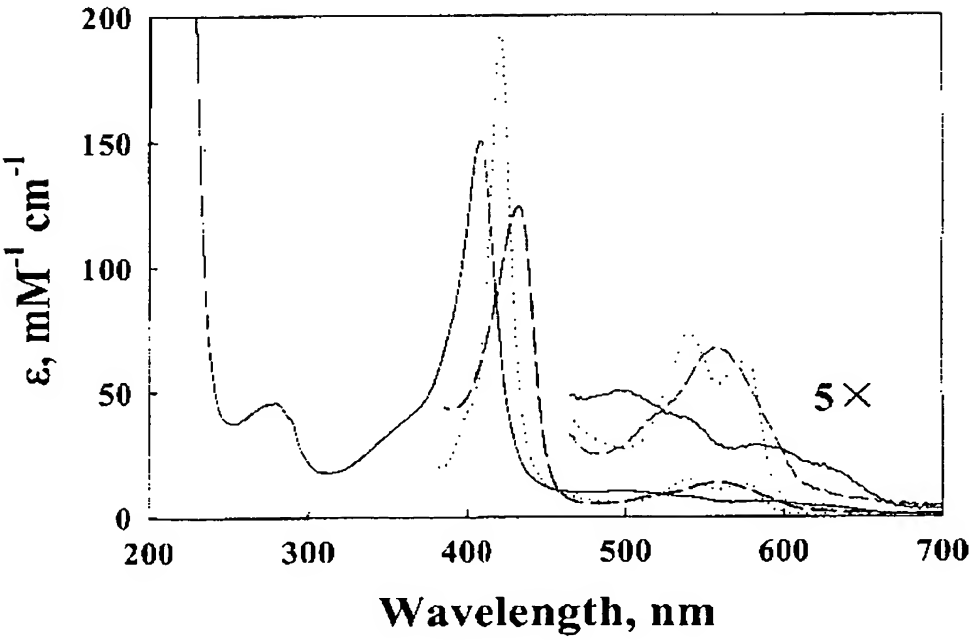
30
20.1
14.4

{Fig. 4}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300
400
500
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Wavelength, nm

{Document name} Abstract

{Abstract}

{Problem} The present invention provides a porphyrin compound containing a biotinyl group that can purify small amounts of hemoprotein in the living body rapidly and simply; a purification method for hemoprotein utilizing such a porphyrin compound containing a biotinyl group; a labeling reagent for hemoprotein; a diagnostic agent for hemoprotein-associated diseases utilizing that reagent; and a therapeutic drug for photodynamic therapy.

{Means for solving the problems} The present invention provides a porphyrin compound containing a biotinyl group represented by Formula (I):

Por-A-Bi

wherein Por represents a porphyrin residue optionally forming a metal complex; Bi represents an optionally substituted biotinyl group; and A represents a C_1-C_{20} hydrocarbyl group, or a heterohydrocarbyl group having 1-5 heteroatoms selected from a group consisting of oxygen, sulfur, and nitrogen, and having 1-20 atoms in total; a method for purifying hemoprotein which use the porphyrin compound; a hemoprotein labeling reagent; a diagnostic agent for hemoprotein associated diseases which use the porphyrin compound; and a therapeutic agent for photodynamic therapy.

{Selected Fig.} None

? t 1/5/all

1/5/1 (Item 1 from file: 351) Links
Fulltext available through: Order File History
Derwent WPI

0008504263
WPI Acc no: 1998-034980/199804
Related WPI Acc No: 2000-071652
XRAM Acc no: C1998-011893
XRPX Acc No: N1998-028069
Use of proto-porphyrin compounds - as labels or light output enhancers in entity quantification procedures
Patent Assignee: PACKARD INSTR BV (PACB)
Inventor: CHRIS R; ROELANT C

Patent Family (3 patents, 20 & countries)

Patent Number	Kind	Date	Application Number	Kind	Date	Update	Type
EP 812920	A1	19971217	EP 1996201674	A	19960614	199804	B
JP 11160313	A	19990618	JP 1997298608	A	19971030	199935	NCE
US 5998128	A	19991207	US 1997876093	A	19970613	200004	E

Priority Applications (no., kind, date): EP 1996201674 A 19960614; JP 1997298608 A 19971030

Patent Details

Patent Number	Kind	Lan	Pgs	Draw	Filing Notes
EP 812920	A1	EN	27	8	
Regional Designated States,Original	AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE				
JP 11160313	A	JA	14		

Alerting Abstract EP A1
A method (A) for quantifying entities comprises:
(a) mixing a porphyrin or protoporphyrin of formula (I) with a sample which is suspected of containing entities to be detected;
(b) collecting the (I)-containing complexes formed, and
(c) detecting and quantifying the collected complexes.
R1 = CH(OH)Me, CH=CH2, Et, H, COMe, CHO, CH(OH)CH2OH or CH=CHO2H(?);
R2 = 1-3C alkyl (especially methyl);
R3 = aryl or aralkyl (especially phenyl);
M = a metal selected from Fe, Co, Ga, Sn, Zn, Cr, Mg, Ni, Ge and Cu.
Also claimed are:
(1) a luminol-type chemiluminescent composition, comprising a compound (I) and an active oxygen providing source.
(2) an adhesion or binding assay, comprising:
(a) providing a suspension of entities to be tested;
(b) mixing >= 1 compound of formula (I) with the suspension, to form complexes with the entities to be tested;
(c) removing excess compound (I), especially by centrifugation, magnetic separation or filtration;
(d) incubating the complexed entities with a target surface to adhere the complexed entities;
(e) removing non-adhering material, and
(f) detecting the adhered complexes.
(3) a kit for quantification of porphyrins, comprising 0.2 M borate buffer, pH 10.3 containing minimal 100 muM perborate, 25 muM luminol, 62.5 muM Fe-EDTA, and a positive control sample containing a porphyrin of formula (I).
(4) a kit for enhancement of the light output obtained with oxidase enzyme systems, comprising 0.2 M borate buffer, pH 10.3 containing < 25 muM perborate, 25 muM luminol, 62.5 muM Fe-EDTA, and a positive control sample containing the enzyme of the oxidase enzyme system.
(5) use of a compound of formula (I) as a universal label which irreversibly binds to all types of surfaces (including molecules, cells, viruses, particles and beads) and which is detectable by luminescence or chemiluminescence, fluorescence and/or radioisotopic techniques.
USE - (I) can be used as universal labels, which can bind (or be attached very strongly to) molecules, particles, beads, microorganisms or cells, without requiring any bridging molecules.
USE - (I) can increase the light output of a luminol-type chemiluminescence composition containing an oxidase enzyme system.
(I) can thus be used in quantitative and/or qualitative analysis of chemical and biochemical compounds. It can be used in

immunoassays and hybridisation reactions.
ADVANTAGE - (I) provide long-lived chemiluminescent detectable products.

Title Terms /Index Terms/Additional Words: PROTO; PORPHYRIN; COMPOUND; LABEL; LIGHT; OUTPUT; ENHANCE; ENTITY; QUANTIFICATION; PROCEDURE

Class Codes

International Patent Classification

IPC	Class Level	Scope	Position	Status	Version Date
G01N-033/532			Main		"Version 7"
C07D-487/22; C09K-011/07			Secondary		"Version 7"
C12Q-0001/26	A	I		R	20060101
G01N-0033/52	A	I		R	20060101
C12Q-0001/26	C	I		R	20060101
G01N-0033/52	C	I		R	20060101

ECLA: C12Q-001/26, G01N-033/52
US Classification, Current Main: 435-004000; Secondary: 435-005000, 435-007210, 435-007320, 435-007330
US Classification, Issued: 4354, 4355, 4357.21, 4357.32, 4357.33

File Segment: CPI; EPI
DWPI Class: B02; B04; D16; S03
Manual Codes (EPI/S-X): S03-E14H
Manual Codes (CPI/A-N): B05-A03; B06-D18; B11-C07; B12-K04; D05-H09; D05-H10

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Ｃ０７Ｄ 487/22		Ｃ０７Ｄ 487/22	
Ｃ０９Ｋ　11/07		Ｃ０９Ｋ　11/07	

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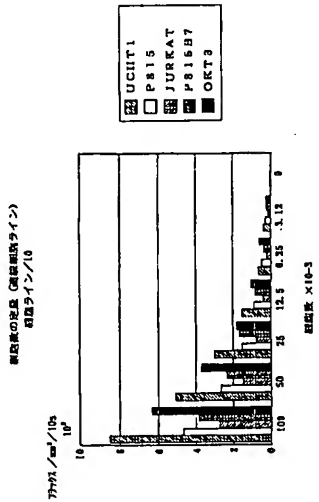
(54)【発明の名称】　ユニバーサル・ラベルとしてのポルフィリン類の使用法

(57)【要約】
【課題】　目標粒子に結合する架橋剤を必要としないで、種々のアッセイと他の定量技術のためのユニバーサル・ラベルとして、ポルフィリン或いはポルフィリン誘導化合物を用いる方法を提供する。
【解決手段】架橋剤なしで、ポルフィリンを検出粒子と、該ポルフィリンが該粒子と結合するに十分な時間混合し：そのラベルされたポルフィリン粒子を非結合ポルフィリンから分け；そして、該ポルフィリンラベルされた粒子を検出する工程を特徴とする該粒子の検出方法。ラベルされた粒子には、ビーズ、微生物、細胞及び分子を含むものである。目標粒子に不可逆的に結合したポルフィリンラベルをその後検出し、ケミルミノ測定、螢光測定或いは放射線測定のような種々の方法により、定量できる。その測定量は、ラベルされた粒子の数に比例するものである。



【特許請求の範囲】
【請求項１】　(a)架橋剤なしで、ポルフィリンを検出粒子と、該ポルフィリンが該粒子と結合するに十分な時間混合し：
(b)ラベルされたポルフィリン粒子を非結合ポルフィリンから分け；そして、
(c)該ポルフィリンラベルされた粒子を検出する工程を特徴とする該粒子の検出方法。
【請求項２】　該ポルフィリンラベルされた粒子は、ケミルミノ測定、放射線測定或いは螢光測定により検出することを特徴とする請求項１に記載の検出方法。
【請求項３】　該ポルフィリンは、プロトポルフィリンIXであることを特徴とする請求項１に記載の検出方法。
【請求項４】　該ポルフィリンは、フェロポルフィリンであることを特徴とする請求項１に記載の検出方法。
【請求項５】　該フェロポルフィリンは、ヘマチン或いはヘミンであることを特徴とする請求項４に記載の検出方法。
【請求項６】　該ポルフィリンラベルされた粒子の定量は、ケミルミノメトリック法により行われ、螢光プルーブと酸化剤を、分離されたポルフィリンラベルされた粒子と混合することを特徴とする請求項１に記載の検出方法。
【請求項７】　該螢光プルーブは、２，３－ジヒドロ－１，４－フタラジンジオンであることを特徴とする請求項６に記載の検出方法。
【請求項８】　該酸化剤は、パーボレイト、水素過酸化物、ヒドロパーオキシド及び酸化剤生産酵素よりなる群から選択されることを特徴とする請求項６に記載の検出方法。
【請求項９】　該ケミルミノメトリック方法を更に、キレート剤とｐＨ保持のための緩衝剤の存在下で行うことを特徴とする請求項６に記載の検出方法。
【請求項１０】　キレート剤は、ＥＤＴＡ或いはデスフェリオキサミンであることを特徴とする請求項９に記載の検出方法。
【請求項１１】　該緩衝剤は、ボレート、カーボネート、トリス（ヒドロキシメチル）アミノメタン或いは燐酸塩緩衝剤であることを特徴とする請求項９に記載の検出方法。
【請求項１２】　該ポルフィリンは、アイソトープ・ラベルされたポルフィリンであり、ポルフィリンラベルされた粒子の検出は、放射線測定方法で行うことを特徴とする請求項１に記載の検出方法。
【請求項１３】　該ポルフィリンは、炭素－１４、塩素－３６、コバルト－（５７、５８、６０）、ガドリニウム－１５３、鉄－（５５、５９）、ニッケル－６３、沃素－１２５、錫－１１３、亜鉛－６５、燐（３２、３３）からなる群から選択される原子でアイソトープ・ラベルされたことを特徴とする請求項１２に記載の検出方法。
【請求項１４】　検出は、放射線測定で行い、ＳＰＡビーズは、沃素－１２５でラベルされたポルフィリンと組合せたものであることを特徴とする請求項１に記載の検出方法。
【請求項１５】　該ポルフィリンラベルされた粒子は、ポルフィリンラベルの螢光の助けにより検出されることを特徴とする請求項１に記載の検出方法。
【請求項１６】　ラベルのために使用されたポルフィリンの量は、約10^{3}Ｍ～10^{5}Ｍであることを特徴とする請求項１に記載の検出方法。
【請求項１７】　該検出粒子は、分子、ビーズ、微生物或いは細胞であることを特徴とする請求項１に記載の検出方法。
【請求項１８】　(a)検出すべき粒子の懸濁液を供給し：
(b)架橋剤なしで、ポルフィリンを検出粒子の懸濁液と混合し：
(c)ラベルされたポルフィリン粒子を非結合ポルフィリンから分け；
(d)該ポルフィリンラベルされた粒子を目標表面で培養し；
(e)非－結合のポルフィリンラベルされた粒子を除去し；そして、
(f)結合ポルフィリンラベルされた粒子を検出する工程を特徴とする結合アッセイ。
【請求項１９】　該ポルフィリンラベルされた粒子を、非結合のポルフィリンから、遠心分離、磁気分離或いは口過により、分離することを特徴とする請求項１８に記載の結合アッセイ。
【請求項２０】　該結合のポルフィリンラベルされた粒子は、放射線測定、螢光測定或いはケミルミノ測定法により検出されることを特徴とする請求項１８に記載の結合アッセイ。
【請求項２１】　(a)検出すべき粒子の予め決めた数の懸濁液を供給し：
(b)架橋剤なしで、ポルフィリンを、該懸濁液と混合し：
(c)ポルフィリンラベルされた粒子を非結合ポルフィリンから除去し；
(d)予め決めた数の粒子により生成した信号を検出し；
(e)粒子の直径或いは表面サイズを表示するものとして、粒子当りの信号を計算する工程を特徴とする表面サイズアッセイの粒子直径。
【請求項２２】　該ポルフィリンラベルされた粒子を、非結合のポルフィリンから、遠心分離、磁気分離或いは口過により、除去することを特徴とする請求項２１に記載の表面サイズアッセイの粒子直径。
【請求項２３】　予め決めた数の粒子により生成され

た信号を、放射線測定、蛍光測定或いはケミルミノ測定法により検出することを特徴とする請求項２１に記載の被面サイズアッセイの粒子直径。

【請求項２４】　(a)研究すべき粒子の懸濁液を供給し：

(b)架橋剤なしで、ポルフィリンを、該懸濁液と混合し：

(c)ポルフィリンラベルされた粒子から、非結合ポルフィリンを除去し；

(d)適当な媒体中にラベルされた粒子を再懸濁し；

(e)ラベルされた粒子及び適当な媒体を生物学的な目標に注入し；そして、

(f)注入された粒子を検出する工程を特徴とする粒子採用研究法。

【請求項２５】　該ポルフィリンラベルされた粒子から、非結合のポルフィリンを、遠心分離、磁気分離或いは口過により、除去することを特徴とする請求項２４に記載の粒子採用研究法。

【請求項２６】　該注入された粒子を、放射線測定、蛍光測定或いはケミルミノ測定法により検出することを特徴とする請求項２４に記載の粒子採用研究法。

【請求項２７】　架橋剤なしで、ポルフィリン含有の第１の容器及びケミルミノ測定、放射線測定、あるいは蛍光測定法によるためのセットを有することを特徴とする粒子検出用アッセイキット。

【請求項２８】　該ポルフィリンは、多重検出アッセイを行う十分な量存在することを特徴とする請求項２７に記載のアッセイキット。

【請求項２９】　蛍光先駆物質及び酸化剤の安定化混合物を含有する第２の容器を有することを特徴とする請求項２７に記載のアッセイキット。

【請求項３０】　該第２の容器は更に、緩衝剤物質及びキレート剤を含有することを特徴とする請求項２９に記載のアッセイキット。

【請求項３１】　該ポルフィリンは、ベーター放射線でアイソトープ・ラベルされ、そのアッセイキットは更に、シンチレイションカクテル含有の第２の容器を有することを特徴とする請求項２７に記載のアッセイキット。

【請求項３２】　ポルフィリン結合粒子を有し、ポルフィリンを該粒子に結合する架橋剤がないことを特徴とする組成物。

【請求項３３】　該粒子は、薬剤、薬剤代謝物、ホルモン、ペプチド、ヌクレオチド、ニュロトランスミッタ、コレステロール、成長因子、オリゴヌクレオチド、抗体、抗原－結合のフラグメント、血清蛋白質、酵素、ポリヌクレオチド、細胞内オルガネラ、細胞表面抗原、アビデン、ビオチン、結合蛋白質、核酸、隔膜プルーブ或いは核酸プルーブであることを特徴とする請求項３２に記載の組成物。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、粒子（例えば、分子、ビーズ、微生物及び細胞）をラベル化、検出し、及び定量する方法に関する。

【０００２】

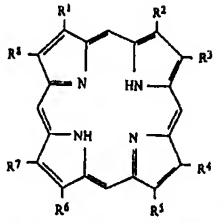
【従来の技術】典型的には、分子、ビーズ、微生物及び細胞を含む粒子を、顕微鏡、ネフェトメトリック或いは電子計数により直接的に検出しそして定量し、或いは、微生物或いは細胞代謝活性の測定により、クロモゲニック或いは蛍光生成染料を使用して、或いは放射活性先駆物質（例えば、放射線金属アイソトープ含有の化合物）を含有させることにより、間接的に検出し、定量しする。検出及び定量のために用いる場合、クロモゲニック或いは蛍光生成の染料及び放射活性先駆物質が、検出され、定量されるべき粒子に結合される。そうであるから、検出され、定量されるべきクロモゲニック或いは蛍光生成の染料及び放射活性先駆物質は、目標の粒子の検出のためのラベルとして役立つ。

【０００３】然し乍ら、クロモゲニック或いは蛍光生成の染料及び放射活性先駆物質により、粒子をラベル化することは、分子或いは官能基と架橋する中間体、或いは目標粒子をラベルに結合する代謝処理を必要とする。これらの方法は、架橋剤を用いるもので、労働力を必要として、よく長期の培養と処理時間を必要とする。

【０００４】種々のアッセイのためのラベルとして、ポルフィリン（或いはポルリン）及びその多くの誘導体を使用することは、先行技術で知られている。ポルフィリンは、テトラピロール顔料マクロサイクルであり、天然で遊離で見いだされるが、金属イオン、典型的には２価金属イオンとの錯体として、よりよく生じる。塩基ポルフィリン構造は、式１に示される。数（１～８）は、官能基が結合し、異種ポルフィリン誘導体を形成できる位置を示す。

【０００５】式１

【化１】

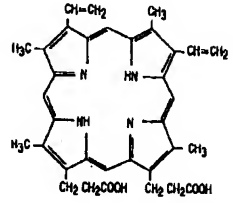


【０００６】種々の誘導体において、個々の基は、数字を添えた置換点各々に存在できる。このような誘導体の例は、各ピロール基のベーター水素がメチル及びエチル基で置換された４種の異性体として存在するアエチオポルフィリンを含むものである。ウロポルフィリンは、酢

酸及びプロピオン酸基をメチル及びエチル基の代わりに用いる点を除いて同様である。コプロプロピレン　ホモ及びコポリマーは同様に４つのメチルと４つのプロピオン酸基を含む。最後の例示の基はプロトポルフィリンであり、４つのメチル基、２つのビニル基と２つのプロピオン酸基を１～８の位置に有する１５の異性体の群である。

【０００７】式２

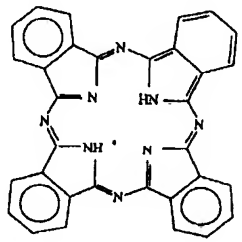
【化２】



【０００８】他の誘導体において、単一置換基が、各ピロール基が数えた位置の両方を占有させる。フタロシアニンは、このタイプの典型的なポルフィリン誘導体である。フタロシアニンもポルフィリン核の４つのピロール基の間を架橋する４つの窒素の存在により特徴づけられる。前記の元のポルフィリン及びその誘導体において、各炭素原子に結合する単一の末端水素原子を有する炭素原子により、この架橋が行われる。式３に示されるものは、フタロシアニンである。

【０００９】式３

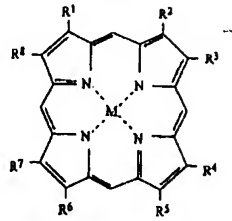
【化３】



【００１０】前記のように、ポルフィリン及びその種々の誘導体は、金属と錯体塩である。これが生じる場合、ピロール基の対角に位置する２つの窒素原子に結合する２つの水素原子が、単一金属原子により置換される。Mは、式４に示される。

【００１１】式４

【化４】



【００１２】典型的な金属、Mは、ポルフィリン構造中に含有できるものは、鉄（Ｆｅ）、コバルト（Ｃｏ）、ガリウム（Ｇａ）、錫（Ｓｎ）、亜鉛（Ｚｎ）、クロム（Ｃｒ）、マグネシウム（Ｍｇ）及びランタン系の種々の元素である。

【００１３】ポルフィリン及びその誘導体（以下ポルフィリンと称する）は、免疫アッセイ、核酸プルーブアッセイ、免疫ブロット、ハイブリッド化アッセイ、顕微鏡観察、イメージ化、流動シトメトリ、ＤＮＡ配列、及びフォトダイナミックの中のラベル（或いはマーカー）として、よく用いられてきた。然し乍ら、ポルフィリン及びその誘導体を、これらの種々のアッセイ及び技術において、粒子のケミルミノ測定法、放射線測定或いは蛍光測定のためのラベルとして、用いるには、架橋剤の使用が必要である。当業界において、架橋剤の使用は、種々の用語で示されるが、それに限定されなく、例えば、架橋（化）、カップル（化）、共有化、結合及びチトラ(tether)すると云われる。分子の反応剤或いは官能基のいずれかである架橋剤は、ポルフィリンラベルを、検出すべき粒子に結合させ、定量できる。ポルフィリンと架橋剤の選択は、目標粒子の特性、目標粒子がある媒体及び用いる検出手段を含む多数の基準に依存して著しく変わる。架橋を与える架橋剤は、例えば、ピロール基のベーター位置の１つを含む可能な位置でポルフィリン上で置換できるものである。そして、それは、ピロール基での置換される基の位置上の末端位置（例えば、フタロシアニンの６－員環の炭素に結合している水素を置換する）、或いはピロール基と結合する炭素での端末位置である。架橋装置がポルフィリン上で置換された位置であるが、結合が生じない位置では溶解度を高めるものである。本当に、置換基は、溶解度高めるためのみにポルフィリン上に提供するものである。

【００１４】多数の架橋剤（例えば、官能基）が、既知である。これらには限定されないが、スルフォン酸基（－SO_3H）、スルフォン酸塩基（－$SO_3^-H^+$）、カンボン酸基（－CO_2H）、カルボキシレート基（－CO_2^{2-},X^+）、燐酸基（－PO_4H_2）、フォスフォネート（－PO_3^{2-},X^+或いは－PO_3H）、ヒドロキシ或いはフェノキシ基（－OH）、アミノ基（－NH_2）及びアンモニウム及びピリジニウム基（－NR_4^+,X^-）がある。更に、架橋を供するに最も普通の方法の１つは、ポ

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ルフィリンの水溶性カルボジイミド誘導体を形成することである。任意に、架橋剤は、ポルフィリンラベルにさらす前に目標粒子に結合できるものである。

【0015】核酸プローブアッセイのような、他の検出方法においては、ポルフィリンラベルを核酸プライマー或いはプローブに結合する必要がある。架橋剤は、ポルフィリンラベル、プライマー或いはプローブ或いは目標粒子のいずれかと先ず結合するもので、ポルフィリンとプライマー或いはプローブとを結合するために用いられる。従って、プライマー或いはプローブはそれ自体が目標粒子に結合する。ポルフィリンラベルを目標粒子に結合するための架橋剤を必要としないポルフィリンラベルを利用する検出方法の必要がある。

【0016】

【発明が解決しようとする課題】本発明は、上記の問題点を解決するためになされたもので、それ自体で、そして、架橋剤なしで、分子ビーズ、微生物及び細胞を含む目標粒子に不可逆的に結合し、その後、ケミルミノメトリック、螢光測定或いは放射線測定のいずれかにより、ラベルされた粒子の数と比較する量で検出できるユニバーサル・ラベルを提供することを目的とする。

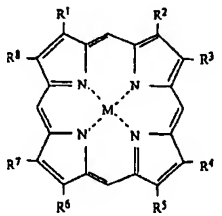
【0017】

【課題を解決するための手段】上記の技術的な課題の解決のためになされたもので、本発明において、目標粒子に結合する架橋剤を必要としないで、種々のアッセイと他の定量技術のためのユニバーサル・ラベルとして、ポルフィリン或いはポルフィリン誘導化合物を用いる方法である。そして、ラベルされた粒子には、ビーズ、微生物、細胞及び分子を含むものである。目標粒子に不可逆的に結合したポルフィリンラベルをその後検出し、ケミルミノ測定、螢光測定或いは放射線測定のような種々の方法により、定量できる。その測定量は、ラベルされた粒子の数に比例するものである。

【0018】ユニバーサル・ラベルのための探求は、ケミルミネセント、螢光或いは放射線活性組成物中のポルフィリンの使用により満足される。特に、本発明は、次式5（式4と同じ構造を示す）のポルフィリンを有するケミルミネセント、螢光或いは放射線活性組成物に関する。

【0019】式5

【化5】



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【0020】式中、R　～R　は、－CH_3、－CH_2-CH_3、－CH=CH_2、－$COCH_3$、－CHO、－CH_2-CH_2OH、－CH=CHOOH或いはフェニルであり得、Mは、鉄（Fe）、コバルト（Co）、ガリウム（Ga）、錫（Sn）、亜鉛（Zn）、クロム（Cr）、マグネシウム（Mg）及びランタン系の種々の元素であり得る。

【0021】1つの面に従うと、本発明は、粒子をラベル化し、検出しそして定量する方法を目的として、その粒子は限定なく、分子、ビーズ、微生物或いは細胞を含み、その工程は次のようである。

(a)ポルフィリンを架橋剤なしでポルフィリンが粒子に結合するに十分な時間混合し；

(b)ポルフィリンラベルされた粒子を非結合のポルフィリンから分離し；そして

(c)該ポルフィリンラベルされた粒子を定量する。

【0022】異種の検出と定量の方法のために、基本的な方法フレームワークは、技術の一般的な処理法に従って変える。ケミルミノメトリック法のために、ポルフィリンラベルされた粒子を、粒子と結合したポルフィリンを不安定化すると螢光を発する、螢光プローブと酸化剤（或いは酸化薬）の安定化混合物に接せしめる。螢光プローブ及び酸化剤の安定化混合物からの発光を検出する。発光量は、ポルフィリンラベルされた粒子の数に比例する。

【0023】放射線測定法のため、粒子に結合したアイソトープ・ラベルされたポルフィリンの放射線発光を検出する。放射線発光の量は、ポルフィリンラベルされた粒子の数に比例する。

【0024】螢光測定法のため、ポルフィリンラベルされた粒子の光励起の後の螢光を検出する。発光量は、ポルフィリンラベルされた粒子の数に比例する。

【0025】他の面では、本発明は、次の工程からなる接着或いは結合アッセイを提供することを目的にする。

(a)試験すべき粒子の懸濁物を供し；

(b)懸濁物とポルフィリンを架橋剤なしで混合し；

(c)遠心分離、磁気分離或いはロ過処理のような方法により過剰のポルフィリンを除去し；

(d)ポルフィリンラベルされた粒子を目標表面に接種し；

(e)非－接着或いは非－結合のラベルされた粒子を除去し；そして

(f)放射線測定、螢光測定或いはケミルミノ測定法のような方法により、表面接着された、或いは結合ラベルされた粒子を定量する。

【0026】他の面では、本発明は、次の工程からなる、粒子直径或いは表面サイズ分析法を提供することを目的にする。

(a)試験すべき粒子の予め決めた数の懸濁物を供し；

(b)懸濁物とポルフィリンを架橋剤なしで混合し；

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(c)遠心分離、磁気分離或いはロ過処理のような方法により過剰のポルフィリンを除去し；

(d)予め決めた数の粒子から生じる放射線測定、螢光測定或いはケミルミノ測定信号を得；

(e)粒子の直径或いは表面サイズの表示物として粒子当りの信号を計算する。

【0027】他の面では、本発明は、次の工程からなる、試験管内或いは生体内採取研究を提供することを目的にする。

(a)試験すべき粒子の懸濁物を供し；

(b)懸濁物とポルフィリンを架橋剤なしで混合し；

(c)遠心分離、磁気分離或いはロ過処理のような方法により過剰のポルフィリンを除去し；

(d)ポルフィリンラベルされた粒子を適当な媒体中に再懸濁し；

(e)ポルフィリンラベルされた粒子を、生物学的な目標中に注入し；そして

(f)注入された粒子を検出する。

【0028】他の面によると、本発明は、粒子、ビーズ、微生物或いは細胞を定量するためのアッセイキットを提供する。アッセイキット中に含有される元素は、用いるべき検出及び定量方法に依存して；ケミルミノ測定法、放射線測定或いは螢光測定により変わる。すべての場合、アッセイキットは、少なくともポルフィリンラベルを含有する第1の容器よりなる。ケミルミノ測定検出のため、アッセイキットは、ポルフィリンラベルを含有する第1の容器と螢光プローブと酸化剤の安定化混合物を含有する第2の容器とを含む。放射線測定検出のために、アッセイキットは、アイソトープ・ラベルされたポルフィリンを含有する第1の容器とシンチレイションカクテル或いはSPA（シンチレイションプロキシミティアッセイビーズ）のいずれかを含有する第2の容器を含む。螢光測定検出にために、アッセイキットは、ポルフィリンラベルを含有する容器を含む。

【0029】1具体例において、目標表面に結合された、分子、ビーズ、微生物或いは細胞を含む多数のポルフィリンラベルされた粒子を、検出し或いは定量するための方法を提供する。この具体例によると、フェリポルフィリンの効果的量を、目標粒子の水性懸濁物に混合する。遠心分離、磁気分離或いはロ過のいずれかにより、過剰のポルフィリンラベルを除去した後、ラベルされた粒子を、選択した水溶液中に必要な密度に懸濁する。

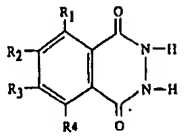
【0030】ラベルされた粒子を次に目標表面に添加し、結合或いは接着するに十分な時間必要な反応状態下に維持する。この後に、用いた条件で、通常に知られるように、変えて、非－結合或いは非－接着のラベルされた粒子を除去する。接着或いは結合されたラベルされた粒子を、ケミルミノ測定法、放射線測定或いは螢光測定を含む所望の検出方法により検出しそして定量する。

【0031】ケミルミノ測定法のために、選択の望ましい水溶液は、十分な量の、螢光プローブ（先駆物質）及び酸化剤の安定化混合物を含有する。得られたケミルミネセンスを、検出しそして定量する。ケミルミネセンスは、ポルフィリンラベルされ、接着され或いは結合された粒子の数に比例する。

【0032】本発明に用いるケミルミネセンス先駆物質は、式6で一般的に示される2，3－ジヒドロー1，4－フタラジンエジオン類である。

【0033】式6

【化6】



【0034】式6中で、R_1は、アミノ基で、R_2、R_3、R_4基は、任意で置換される。特に、ケミルミネセンス先駆物質として好適なものは、5－アミノー2，3－ジヒドロー1，4－フタラジンジオン（ルミノール）である。

【0035】ケミルミノ測定検出方法と組合せた本発明の具体例を行うための変更例において、固体表面（例えば、隔膜、ディップステックス）上に位置されたポルフィリンラベルされた接着或いは結合され、透明な容器中に含まれ、或いは、透明なウエル底でマイクロメッキあれた粒子は、ポラロイドフィルムカートリッヂのような高スピード写真フィルム上に配置される。免疫化のラベルされた粒子は、容器内の螢光プローブ及び酸化剤の安定化混合物を注入することにより検出される。安定化混合物は、螢光プローブ、酸化剤及びポルフィリンラベルされた粒子の混合物の間での反応により、発光する接着或いは結合の粒子に接着させられる。替わりに、生じた光は、フォトマルチプライヤ管（PMT）或いはCCDカメラのような既知の技術により検出できる。

【0036】ケミルミノ測定検出が望ましい場合、酸化剤は、ポルフィリンに反応して、ケミルミネセンス先駆物質を励起させ、それにより、螢光反応で発光することを、本発明により利用する。特に好適な酸化剤は、例えば、過酸化水素及びパーボレイトイオンのような過酸化物、或いは酵素反応によりそれ自体で生成するようなものである。

【0037】ケミルミノ測定法において、緩衝物質を用いることが更に、望ましい。用いることができる適する緩衝物質は、燐酸塩、トリス（ヒドロキシメチル）アミノメタン、カーボネイト及びボレイトである。更に、螢光先駆物質及び酸化剤の混合物の安定化は、デフェリオキサミン或いはエチレンジアミンテトラ酢酸（EDTA）のようなキレート剤の添加により得られる。

【0038】螢光先駆物質、安定剤及び酸化剤の次の薬

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剤組成物は、特に、ケミルミノ測定検出方法を用いて、６．５ｍＭのパーボレイト、３．４ｍＭのＥＤＴＡ及び０．１ｍＭのルミノールを含有する０．１Ｍボレイト緩衝剤、ｐＨ９．５０を用いる具体例用に特に適する。

【００３９】効果的な検出量のポルフィリンは、計数すべき粒子の数に比例する検出の蛍光信号を提供するに必要なポルフィリン量である。効果的な検出量は、粒子の数と特性と及びポルフィリン特性の中で変化する。

【００４０】粒子、ビーズ、微生物或いは細胞の数は、約０～約10^{9}／ｍｌであり、蛍光プルーブがルミノールである場合、ラベルのために用いるポルフィリンの効果的検出量は、約10^{3}～約10^{5}Ｍである。

【００４１】ラベル化及び検出の条件は；温度、ｐＨ値、オスモラリテイ、トニシテイ等である。典型的には、温度は、約５℃～約５０℃の範囲で、好適には、約２０℃～約４０℃の範囲である。ラベル化のためのｐＨは、約６～約８．５で、好適には約６．５～約７．５の範囲である。検出ｐＨは、約７．５～約１２．５であり、好適には約８～約１０．５の値の範囲である。ポルフィリンラベル化の保持時間は、約５～約２０分間であり、好適には約１０分間である。

【００４２】放射線測定方法のために、粒子に結合したアイソトープ・ラベルされたポルフィリンの放射線発光を、検出し、定量する。発した放射線量は、ポルフィリンラベルされた粒子の数に比例する。選択の所望の水溶液は、十分な量の適するシンチレイションカクテルを含有する。目標粒子が、ポルフィリンラベルを含有するアイソトープ（ベーター線マーカー）に結合している。発した放射線を、ＰＭＴ或いはＣＣＤカメラにより検出する。代替的に、アイソトープ・ラベルされたポルフィリンでラベルされた、そして、適する固体シンチレイターで被膜した表面に接着され、或いは、結合された粒子の、発した放射線は、直接的にＰＭＴにより検出される。

【００４３】放射線測定技術に用いられる適当なベーター線発光のアイソトープには、炭素－１４、塩素－３６、コバルト－（５７、５８、６０）、ガドリウム－１５３、鉄－（５５、５９）、ニッケル－６３、トリチウム、沃素－１２５、錫－１１３或いは亜鉛－６５がある。

【００４４】蛍光測定法にために、接着性のラベルされた粒子を、波長がポルフィリンラベルの励起波長に合致する光線にさらす。ポルフィリンラベルの励起の後或いはその間に、励起されたポルフィリンによる発光が、ＣＣＤカメラ或いはＰＭＴにより検出される。発光量は、ポルフィリンラベルされた粒子の数と比例する。

【００４５】本発明の具体例により、目標粒子として利用できるビーズの非限定的な例は、ナイロン、プラスチック、ポリスチレン、ポリプロピレン、ラテックス、ガラスにより作られた粒子或いはビーズを含む。ビーズ

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は、リポソーム（細胞内脂肪粒子）を含む固体球体或いは空洞球体のいずれかを意味し、その大きさは、サブミクロスコープから約１ｃｍの範囲である。更に、これらの粒子或いはビーズは、リガンド、ハプテン或いはビオチン、ビオチン－Ｎ－ヒドロキシサクシニミド或いは抗体、アビジン及びストレプタビジン等の結合蛋白質のような架橋分子を有するものである。本発明の具体例により、目標粒子として利用できる細胞の例として、限定されないが、プロカリオテック及びエウカリオテック及び哺乳動物細胞タイプを含むものである。ここで使用できるように、粒子は、不定形の粒子相を意味し、分子、ミセラス及びコロイドを含み、粒子の大きさは、サブミクロンから約１ｃｍまでの範囲である。

【００４６】前記の方法の適用は非常に広いことは明らかになった。ポルフィリンが以前からラベルとして使用された粒子は、前記の方法により利用できる。例えば、米国特許第５，４９４，７９３号を参照。この指標として、次のものがあるが、それに限定されなく、その検出され得る、粒子のリストには、薬剤、薬剤代謝物、ホルモン類、ペプチド、ヌクレオチド、神経伝達物質、コレステロール、成長因子、オリゴヌクレオチド、抗体、抗原－結合フラグメント、血清蛋白質、酵素、ポリヌクレオチド、細胞内オルガネラ、細胞表面抗原、アビジン、ビオチン、結合蛋白質、核酸、隔膜プルーブ及び核酸プルーブがある。

【００４７】この方法は、多数の目的のために、従来の用いたラベルの検出技術のために利用できる。例えば、米国特許第５，４９４，７９３号、第５，３４０，７１４号、第５，３０６，６２４号、第４，９９４，３７３号、第４，６５９，６７６号、第４，６１４，７２３号及び第４，４８５，０８６号を参照。方法に使用できる技術は、これに限定されないが、競合的、置換法或いはサンドウイッチ免疫アッセイ、核酸プルーブアッセイ、免疫ブロット法、ハイブリットアッセイ、顕微鏡観察；イメージ法、流動シトメトリイ、ＤＮＡ配列及びフォトダイナミック治療法がある。

【００４８】本発明の具体例で利用できるポルフィリンの限定しない例は、金属錯体化したプロトポルフィリンを含む。好適な金属プロトポルフィリンは、フェリーポルフィリン、特にフェロプロトポルフィリンＩＸであり、これから誘導されたポルフィリン類である。このポルフィリン－誘導の構造は、式７に示され、ヘモグロビン、ミオグロビン、エリトロクルオリン、カタラーゼ、ペルオキシダーゼ及びクラスＢのシルクロームである。

【００４９】式７

【化７】

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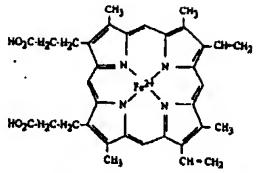
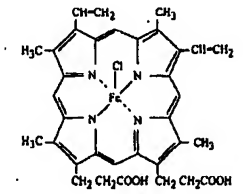


【００５０】特に、フェロプロトポルフィリン例えば、クロロヘミン（式８）或いはヘマチン（式９）は、目標粒子に非常に効率よく添加され、そして、非常に効率よく蛍光発光させ、或いは、説明のように、ケミルミネセントを発する例である。

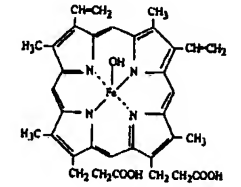
【００５１】式８

【化８】



【００５２】式９

【化９】



【００５３】他の具体例において、ＳＰＡ（シンチレイション　プロキシミテイ　アッセイ）ビーズ、接着物或いは懸濁物中で検出し、そして定量する方法は、次の工程を有する。

(a)アイソトープ・ラベルされたポルフィリンを、検出され、定量されるに必要なＳＰＡビーズがある水性懸濁物と混合し；

(b)放射線発光の上昇を測定する。

この場合の放射線発光の上昇は、懸濁物中に存在するビーズの数に比例する。

【００５４】また他の具体例において、ビーズ、微生物、細胞及び分子を含む、粒子を検出しそして定量するためのアッセイキットを提供する。アッセイキット内容物は、用いる検出方法に依存して変えるが、一般的に、ポルフィリンラベルを含有する第１容器を含む。放射線測定検出が望ましい場合、ポルフィリンは、適当なベータ発光アイソトープでラベルされるべきである。ポルフィリンラベルは、適当な媒体中に懸濁でき、或いは溶解

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でき、或いは、乾燥形にできる。処方物上のみの制限は、特定処方物がラベルに安定性を与え、ラベルが貯蔵中に化学的に変更しないようにするものである。

【００５５】更に、アッセイキットは、用いるべき検出方法に依存して変化する内容物を有する第２容器もある。ケミルミノ測定検出が望ましい場合、第２の容器は、少なくとも１つの定量アッセイを行うに十分な量の、蛍光先駆物質と酸化剤の安定化混合物を含有する。安定化混合物は、ビーズ、微生物、分子及び細胞を含む粒子と干渉し、粒子の数に比例する量の第１容器のポルフィリンラベルでラベルされる。蛍光先駆物質及び酸化剤の安定化混合物が、懸濁物、溶液として、乾燥形で（例えば、錠剤）として存在できる。適当な緩衝剤或いはキレート剤もまた存在できる。

【００５６】放射線測定のために、第２の容器は、ベーター線発光物でアイソトープ・ラベルされたポルフィリンを検出するための適当なシンチレイションカクテイルを含有する。シンチレイションカクテイルは、少なくとも１つの定量アッセイを行うに十分な量で存在する。

【００５７】具体例により、ケミルミノ測定のために適する、例示のキットは、ジメチルスルフォキシド（ＤＭＳＯ）中の１．５ｍＭのヘマチン（ポルフィリンラベル）１ｍｌを含有する第１容器と、６．５ｍＭのナトリウムパーボレイト（酸化剤）１１０ｍｌ、安定化剤として３．５ｍＭのＥＤＴＡ及びｐＨ９．５の０．１Ｍボレイト緩衝剤中の０．１ｍＭのルミノール（蛍光先駆物質）を含有する第２容器を有するものである。約４℃で適当に貯蔵されると、これらの溶液は、数ケ月安定にある。

【００５８】好適な具体例によると、第１及び第２の容器は、含有する成分の特性、量或いは濃度及び効果的量を示す指標でラベルされる。

【００５９】他の具体例において、説明される、ケミルミノ測定、放射測定及び蛍光測定技術は、目標粒子の粒子直径或いは表面サイズを測定するために用いることができる。これは、既知の量の粒子を、前記の技術の１つにより用いることにより達成される。粒子当りの検出信号の量は、粒子の直径或いはサイズを指標するものである。

【００６０】他の具体例において、説明される、ケミルミノ測定、放射測定及び蛍光測定技術は、試験管内及び生体内研究のために用いることができる。この目的のために、ポルフィリンラベルされた粒子は、通例のポルフィリンから分離され、適当な媒体中に再懸濁され、目標の生物学的目標中に注入する。ラベルされたラインを、次に、ケミルミノ測定、放射線測定及び蛍光測定技術により調べる。

【００６１】次に、本発明を本発明の特定の具体例により説明するが、本発明はそれらによって限定されるものではない。

【0062】

【実施例1】［細胞数（連続細胞ライン）の定量］

材料と方法

UCHT1、P815、ジャカットT－細胞、P815B7及びOKT3細胞は、回収され、遠心分離にかけられ、そして、ドルベッコ(Dulbecco)のPBS中に、1．10^6細胞／mlの密度で標準的15ml ファルコン管中で再懸濁化された。

【0063】他の柔かに混合した後、細胞を室温で更に5分間法と放置した。150gで、10分間遠心分離にかけた後に、細胞ペレットを更なる4mlのPBS中に再懸濁し、そして、再び、洗浄し、すべての過剰のヘマチンラベルを除去できた。

【0064】最後に、細胞ペレットを10^6／mlの密度で再懸濁した。次に、細胞を、白色マイクロタイター皿の個々の壁中に置いて、細胞数は、約100μlのPBS／壁の全量で、約0細胞／壁～約100，000細胞／壁の範囲である。

【0065】ケミルミネセントの検出

次に、100μlの安定化ルミノ／パーボレイト組成物（6．5mMパーレイト、3．4mMのEDTA及び0．1mMのルミノールを含有する0．1Mボレイト緩衝剤pH9．5を各々の壁に添加した。10分後に生成されたケミルミネセンス（フラッスス／mm^2／10秒）が、電荷された結合器（CCD）カメラを使用して、室温で記録された。結果は図1に示される。

【0066】

【実施例2】［細胞数（単離細胞）の定量］

マウスのプロシアルラバグにより単離されたマクロファージをプールし、10^5／mlの密度でダルベッコのPBS中で洗浄した後に濃縮した。次に、10μlのヘマチン（DMSO中の1mg／ml）を、1mlのマクロファージ懸濁物に添加した。細胞を培養し、上記のように、洗浄した。

【0067】100μlの安定化ルミノール／酸化剤組成物の細胞に接触させた10分後に発射されたケミルミネセンス(フラックス／mm^2／10秒）を検出した。結果を図2に示す。

【0068】図1及び2は、ヘマチンラベルされた細胞で観察されるケミルミネセンスは、細胞の数に比例する。図はまた、連続細胞ラインのラベル化及び単離細胞のラベル化が可能であることを示している。

【0069】

【実施例3】［不活性粒子の定量］

不活性粒子の例として、Dynal A.S.,N-0210 Oslo.ノルウエイから、$2.\ 10^8$ビーズ／mlの懸濁物として市販される非被膜のダイナビーズDynabeads M-450を洗浄し、ダルベッコのPBS中の10^7ビーズ／mlの密度で懸濁した。10μlのハマチン貯蔵溶液（DMSO中の1mg／ml）を添加した後に、ロトラック上でゆっくりと混合しながら、ビーズを室温で10分間培養した。培養10分の後に、ビーズを磁気分離法により培養混合物から分離し、PBSで2回洗浄し、最後に、PBS中の10^7ビーズ／mlの密度に再懸濁した。

【0070】最後に、ラベルされたビーズを、白色96ウエルマイクロタイター皿の個々のウエル中にメッキした、ビーズ数は、約100μlのPBS／ウエルの全量において、約0ビーズ／ウエル～約10^6ビーズ／ウエルの範囲である。

【0071】次に、ケミルミネセンス（フラックス／mm^2／10秒）が、前記のように、100μl安定化ルミノール／酸化剤溶液を添加することにより、検出された。この実験の結果は、図3に示される。

【0072】

【実施例4】［微生物の定量］

10^8のスタフィロコッシアウレウア(Staphylococci aureus)の懸濁物をダルベッコのPBS中に、Trypcase Soya Brothトリペキャースソヤブロス中に、一昼夜成長させたスタフィロコッシアウレウアの粗製懸濁物から、調製した。

【0073】洗浄後、1mlのスタフィロコッシアウレウアの懸濁物を、10μlのヘマチン貯蔵溶液（DMSO中の1mg／ml）添加により実施例3のようにラベル化した。

【0074】室温で10分間した後に、スタフィロコッシアウレウアの懸濁物を遠心分離（（450g／5分）し、バクテリアペレットを5mlのPBSで洗浄した。この洗浄処理を2回して、その後ラベルされたバクテリアのペレットを10^8バクテリア／ml PBSの密度に再懸濁した。

【0075】次に、バクテリア希釈液を、100μl PBSの全量での0～10^8バクテリアの範囲の白色マイクロタイター皿のウエル中に調製した。

【0076】ケミルミネセンス（フラックス／mm^2／10s）が開始され、上記の実施例3のように検出された。結果を図4に示す。

【0077】

【実施例5］［ナーザル細胞モノレイヤに異なる株の結合バクテリア］

2種株のスタフィロコッシアウレウアの懸濁物（A，B）を、PBS中の10^9／mlの密度で製造し、実施例4のように、ヘマチンでラベルされた。100μlの全量中で、10^8、5×10^7、$2.\ 5\times10^7$及び0のバクテリアを含有する4つの試料を、合同の成長の接着ヒト ナーザルエピテリアル細胞モノレイヤで培養した。湿化した培養器（空気、5%CO_2）中で37℃1．5時間培養した後、非接着のバクテリアを、ゆっくり洗浄することにより除去した。次に、100μlの、ダルベッコのPBSを、測定すべきウエルに添加した。ケミルミネセンス（フラックス／mm^2／10s）を開始し、実施例4のように測定した。結果を図5に示す。それは、異なるスタフィロコッシアウレウア株の異なる接着性があることを示す。図5に示すように、スタフィロコッシアウレウアA或いはBのいずれか100%を含有する試料のケミルミネセンスがある。

【0078】

【実施例6］［フィブロネチン被膜マイクロタイターウエル上のPMA刺激CD4＋T－細胞の結合］

CD4＋T－細胞を、最初にフィコリーハイパック(Ficoll-Hypaque)傾斜物の上で先ず遠心分離をしたヒト全血試料からの標準処理法により、T－細胞調製物から単離した。CD4＋T－細胞を、ダイナビーズを用いて磁気分離により単離した。

【0079】最後に、CD4＋T－細胞を、PBS中の10^6／mlの最終濃度に懸濁させる。10μlのヘマチン（DMSO中の1mg／ml）を添加し、実施例2に示すようにT－細胞をラベル化した。

【0080】ラベル化し、洗浄した後に、CD4＋T－細胞を、50%（V／V）PBS／ハンクス アルブミン（0．1%）中で10^6／mlの密度で再懸濁した。

【0081】平行して、CD4＋T－細胞の他のアリコットを、標準的プロトコールにより^{51}Crラベルされた。

【0082】次に、フィブロネクチン被膜（FC）或いは非－フィロネクチン被膜（NFC）のどちらかである白色マイクロチアター皿のウエルを3倍化するために、50μlのPBS／HSAを添加し、或いは、10^{-6}フォルボール ミリステート酢酸塩（PMA）を含有する50μlのPBA／HSAのいずれかを添加した。便宜のために、4つの可能な状態は、PMAが存在しない場合、FC及びNFCと称され、PMAが存在する場合PCw／PMA及びNFCw／PMAと称される。

【0083】次に、50μlヘマチンラベルされたT－細胞或いは^{51}CrラベルされたT－細胞を異なるウエル組成物に添加した。2時間37℃（湿気空気、5%CO_2）培養した後、非－接着細胞を徐々に除去した。

【0084】次に、^{51}Crラベルされた細胞を、100μlのトリトンX－100溶解溶液を用いて、溶解せしめ、ガンマ計数により放射線活性を測定した。

【0085】非－接着のヘマチンラベルされたCD4＋T－細胞を除去した後、100μlのPBSをウエルに添加し、そして、100μlの前記の安定化ルミノール／酸化剤溶液を添加した後に、ケミルミネセンス（フラックス／mm^2／10s）を10分間測定した。その結果は図6に示す。

【0086】

【実施例7］［ヘマチンラベル化及びビールス生産細胞ライン］

DSN非ビールス生成及びDSNpJD214MDR1感染及び細胞モノマー生成細胞が、ペトリ皿（ファルコンFalcon)中で合流して成長され、10mlのダルベッコ(Dulbecco)のPBS中に10分間含有した100μgのヘマチンでラベルされた。ラベル化した後、細胞を2回、過剰PBSで洗浄した。対照物は、PBSがヘマチンラベルを含有しないで、同様に製造された。

【0087】次に、10mlの1MDM（胎牛血清なしで）を、皿に添加し、次に、一昼夜培養した。

【0088】次の日に、10μlの上澄み液を各皿から採用し、白色のマイクロタイター皿のウエルに移転した。100μmの前記の安定化ルミノール／酸化剤の溶液を添加し、ケミルミネセンスを10分後に記録した。

【0089】結果は、図7に示される。

【0090】顕著な信号は、対照液（ラベルなし）の上澄み液では生成されない。然し乍ら、ケミルミネセンスでの顕著な値が、ラベルされたが非－ビールス生成ラインから得た上澄み液、及び、ラベルされたビールス－生成モノレイヤーからの上澄み液で見られた。

【0091】後者から誘導された上澄み液で見られたケミルミネセンスの上昇から、本発明がビールス発芽方法を検出することを可能にすることが示唆される。

【0092】最後に、ラベルされなく、非－生成のDSN細胞のモノレイヤーを、ラベルされたpjD214ビールス生成細胞モノレイヤーの上澄み液で培養した場合、ルミノ／酸化剤の溶液の添加の後に、ケミルミネセンスの上昇が、最初のラグ相の後の洗浄のpjD214細胞モノレイヤーで観察された。

【0093】図8に示されるこれらの結果から、安定化のルミノール／酸化剤の溶液が、ヘマチン－ラベルされたビールスをDSN細胞内で検出することが示唆される。約10分間の観察されたラグー相により、ルミノ／酸化剤の溶液が、ビールスにより選ばれたヘマチンにより徐々に不安定化される前に、ゆっくりと細胞内に浸透することが示唆される。

【0094】

【発明の効果】本発明の検出方法は、次のごとき技術的効果があった。即ち、第1に、目標粒子に結合する架橋剤を必要としないで、種々のアッセイと他の定量技術のためのユニバーサル・ラベルとして、ポルフィリン或いはポルフィリン誘導化合物を使用できる。

【0095】第2に、ラベルされた粒子には、ビーズ、微生物、細胞及び分子についても検出でき、定量できる。第3に、目標粒子に不可逆的に結合したポルフィリンラベルをそのまま、検出でき、ケミルミノ測定、螢光測定或いは放射線測定のような種々の方法により、定量できる方法が提供する。そして、第4に、本発明の測定量は、ラベルされた粒子の数に比例するものである。

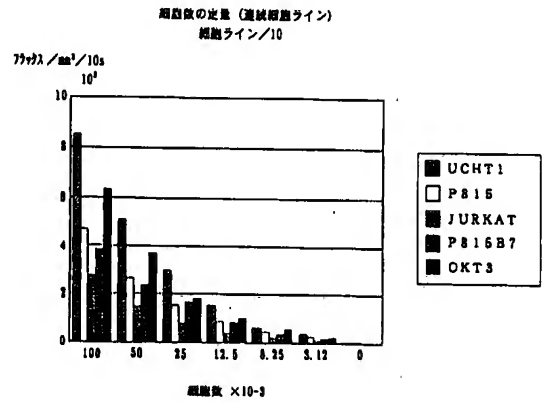
【図面の簡単な説明】

【図1】本発明の検出方法による細胞数の定量したグラフである。

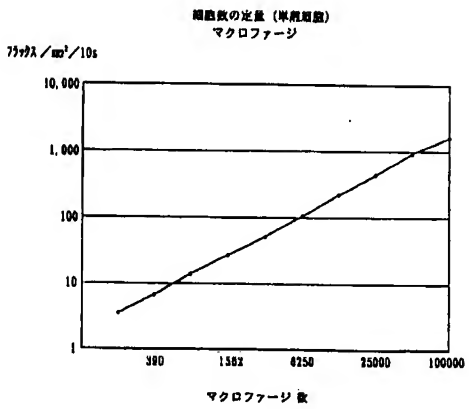
【図2】本発明の検出方法による単離細胞数とフラック

ス密度と関係を示すグラフである。
【図３】本発明の検出方法による不活性粒子数とフラックス密度と関係を示すグラフである。
【図４】本発明の検出方法による微生物数とフラックス密度と関係を示すグラフである。
【図５】本発明の検出方法による、モノレイヤに接着した微生物数とフラックス密度と関係を示すグラフである。
【図６】本発明の検出方法による、Ｔ－細胞数とフラックス密度と関係を示すグラフである。
【図７】本発明の検出方法による、ビールス数とフラックス密度と関係を示すグラフである。
【図８】本発明の検出方法により、ラベルされたビールスで感染した細胞を時間にプロットしたグラフである。

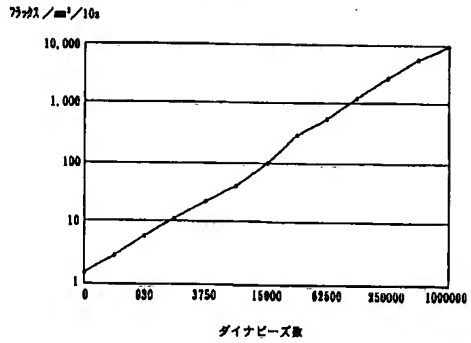
【図１】



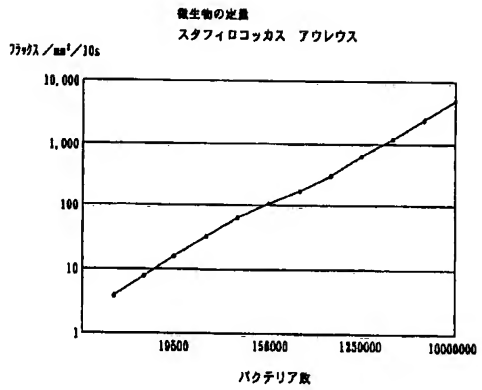
【図２】



【図３】

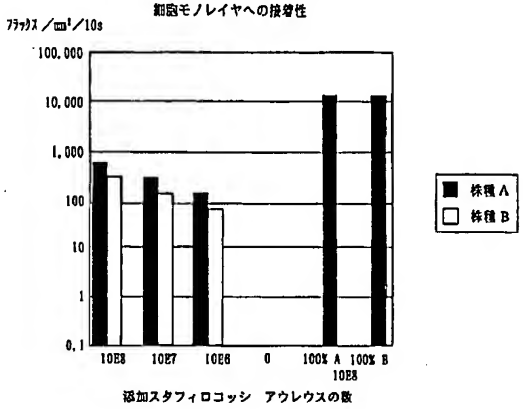


【図４】



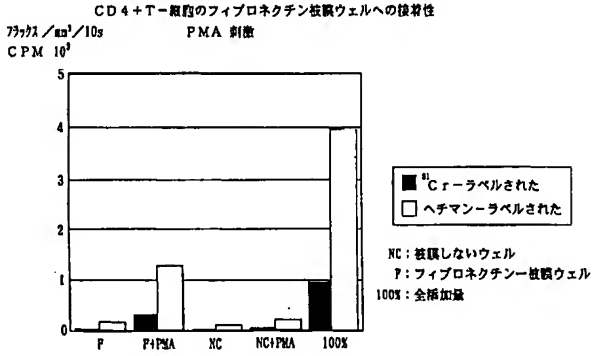
【図５】

スタフィロコッシ　アウレウスの
細胞モノレイヤへの接着性



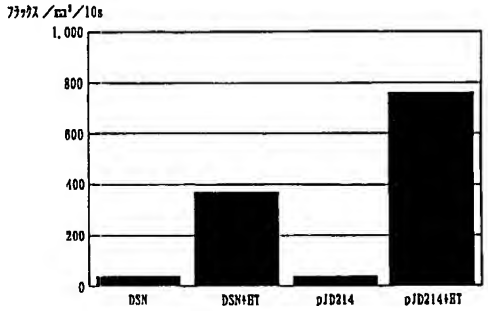
【図６】

CD４＋T－細胞のフィブロネクチン被膜ウェルへの接着性



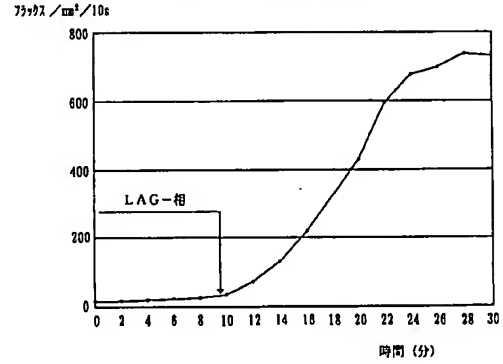
【図７】

ユニライト　ピールス　ラベル
DSN pJD214MDR1



【図８】

ラベルされたピールスに感染された細胞



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(54) Title: MOLECULAR LINKERS SUITABLE FOR CRYSTALLIZATION AND STRUCTURAL ANALYSIS OF MOLECULES OF INTEREST, METHOD OF USING SAME, AND METHODS OF PURIFYING G PROTEIN-COUPLED RECEPTORS

(57) Abstract: A method of crystallizing a molecule-of-interest is disclosed. The method comprises (a) contacting molecules of the molecule-of-interest with at least one type of heterologous molecular linker being capable of interlinking at least two molecules of said molecule-of-interest to thereby form a crystallizable molecular complex of defined geometry; and (b) subjecting said crystallizable molecular complex to crystallization-inducing conditions, thereby generating the crystal containing said molecule-of-interest.

MOLECULAR LINKERS SUITABLE FOR CRYSTALLIZATION AND STRUCTURAL ANALYSIS OF MOLECULES OF INTEREST, METHOD OF USING SAME, AND METHODS OF PURIFYING G PROTEIN-COUPLED RECEPTORS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to molecular linkers suitable for crystallization and structural analysis of molecules of interest, to method of using same, and to methods of purifying G protein coupled receptors (GPCRs). More particularly, the present invention relates to methods of crystallizing membrane proteins and to methods of purifying GPCRs via affinity chromatography using arrestin derived polypeptides.

Importance of protein structure determination: The recently fully sequenced human genome, has been found to contain up to 38,000 genes (Venter JC. *et al.*, 2001. Science 291:1304) encoding up to an order of magnitude more protein species. It is evident that the information contained therein holds tremendous potential for furthering the development of practical applications in all fields involving the life sciences. However, most proteins remain to be characterized with respect to their structure and function and, although the transcription profiles of the genes encoding these proteins are currently being determined, such data can yield only limited information. In order to fully harness the potential of the information contained in the complete human genome sequence, it will be necessary to systematically determine the three-dimensional (3D) structure of the proteins encoded therein.

The capacity to solve the 3D atomic structure of proteins is proving to be crucial for understanding and regulating their biological functions and, as such, is playing an increasingly vital role in the advancement of biomedical science and biotechnology, in particular in the realm of drug design.

The pathogenesis of a very large number of human diseases involves membrane proteins such as GPCRs, as startlingly demonstrated by the fact that a 60 % majority of approved drugs elicit their therapeutic effects by selectively targeting members of the GPCR family (GlaxoWellcome, 1996. Nature Suppl.

384:1-5). However, pharmacological treatment of diseases involving GPCRs remains far from optimal and there is thus a critical need for novel and improved GPCR-targeting drugs. As highlighted, for example, by the 3D atomic structure-based development of protease inhibitors employed in the first effective treatment of human immunodeficiency virus (HIV) induced acquired immuno-deficiency syndrome (AIDS) (Wlodawer A. and Vondrasek J., 1998. Annu Rev Biophys Biomol Struct. 27:249), the development of novel and improved membrane protein-targeting drugs, such as GPCR-targeting drugs, can dramatically benefit from the availability of the 3D atomic structure of such drug targets.

Other increasingly important applications of protein crystals include their use as catalysts on a commercial scale, in bioremediation and green chemistry applications, and in purification-related applications, such as enantioselective chromatography of pharmaceuticals and high-grade chemicals. In the near future, their utility will further expand to include the purification of protein drugs and the development of adjuvant-less vaccines (Margolin AL. and Navia MA., 2001. Angewandte Chemic International Edition 40:2204).

General obstacles to protein crystallization: The bottleneck in determination of novel protein structures has shifted from the collection and interpretation of crystallographic data to the production of large amounts of highly pure protein and the generation of diffraction-grade crystals. Techniques for growing such crystals currently rely substantially on empirical processes for which only general rules of thumb are available and which frequently require adaptations tailored to accommodate the peculiarities of individual proteins.

Several factors contribute to the difficulty in obtaining highly ordered protein crystals. Although contacts between crystallized protein molecules are of comparable energy to those between small molecules, the significantly fewer number of intermolecular contacts per molecular weight of crystallized protein molecules renders these contacts very fragile (Carugo O. and Argos P., 1997. Protein Science 6:2261). Furthermore, due to their inherent complexity, protein

molecules can assume numerous conformations, a phenomenon which tends to prevent formation of highly ordered crystals. Moreover, aggregated proteins are able to form many different types of intermolecular contacts of which only a restricted number will generate highly ordered crystals. Hence, crystallization conditions must be carefully fine-tuned so as to induce the proper molecular conformation and packing orientation of each molecule accreted during the process of crystallization. Such conditions are difficult to obtain since small variations in physico-chemical parameters, such as pH, ionic strength, temperature or contaminants, will strongly influence the process of crystallization in a way that is unique for each protein due to the diversity of the chemical groups and possible configurations thereof involved in the formation of intermolecular contacts (Giege R. *et al.*, Acta Crystallographica Section D-Biological Crystallography 1994. 50:339; Durbin SD. and Feher G., 1996. Annu Rev Phys Chem. 47:171; Weber PC., *Overview of protein crystallization methods*, in *Macromolecular Crystallography, Pt a.* 1997. p. 13-22; Chernov AA., Physics Reports-Review Section of Physics Letters 1997. 288:61; Rosenberger F., *Theoretical and Technological Aspects of Crystal Growth* 1998. p. 241; Wiencek JM., 1999. Annu Rev Biomed Eng. 1:505).

Obstacles to membrane protein crystallization

Three dimensional protein structure determination at high resolution represents a particularly difficult challenge for membrane proteins and the number of such proteins that have been crystallized is still small and far behind that of soluble proteins, even though membrane proteins represent up to 40 % of the proteins encoded by the human genome (Wallin E. and von Heijne G., 1998. Protein Sci. 7:1029).

The crystallization of membrane proteins is particularly difficult due to the fact that, unlike soluble proteins which tend to have hydrophilic surfaces and polar cores, membrane proteins have significant hydrophobic surfaces through which they interact with membrane lipids. Such proteins exist in a quasi-solid state in the membrane and are not readily soluble in either aqueous or apolar

environments.

The most widely employed approach for solubilization of membrane proteins is the use of detergents interacting with the hydrophobic surfaces of the protein to generate mixed detergent/protein micelles. Solubilized membrane proteins can then be crystallized in an ordered two-dimensional (2D) lattice by reconstitution in an artificial lipid bilayer, allowing 2D structural determination via electron microscopy. While such 2D crystals are relatively easy to obtain, the use of electron microscopy for determining molecular structure suffers from the significant drawback of generating structural information with poor resolution in directions orthogonal to the 2D lattice, thus preventing structural determination at sufficiently high resolutions (Stowell MH. *et al.*, 1998. Curr Opin Struct Biol. 8:595). An additional factor contributing to the difficulty of determining the structure of membrane proteins at high resolution is due to the fact that crystal contacts made between detergent micelles tend to be disordered, resulting in poorly diffracting crystals. Although the use of helical crystals and advanced image processing can obviate some of these drawbacks, it is only with X-ray crystallography of 3D crystals that high resolution determination of 3D protein structure can be achieved. This is essential, for example, to generate detailed pictures of molecular target sites when designing drugs specifically interacting with such sites. In the case of membrane proteins, this is highly desirable since such information can significantly contribute to the design and development of novel drugs for the very large number of diseases whose pathogenesis involves membrane proteins, such as receptors. Such diseases include, for example, cancer, viral diseases such as AIDS, neurological disorders, metabolic illnesses such as diabetes, etc.

Prior art optimization of crystallization conditions

High throughput techniques

High throughput techniques are currently being employed to determine the conditions required for growth of protein crystals. One such approach employs automation to perform large numbers of crystallization trials (Morris, DW. *et al.*,

1989. Biotechniques 7:522; Zuk WM. and Ward KB., 1991. Journal of Crystal Growth 110:148; Heinemann U. *et al.*, 2000. Progress in Biophysics & Molecular Biology 73:347).

Such high throughput approaches employ the sparse-matrix protein crystallization method, in which a series of crystallization conditions are tested in parallel, the most promising ones being iteratively refined until crystallization is achieved (Jancarik J. and Kim SH., 1991. Journal of Applied Crystallography 24:409; Cudney B., *et al.*, 1994. Acta Crystallographica Section D-Biological Crystallography 50:414; Hennessy D. *et al.*, 2000. Acta Crystallographica Section D-Biological Crystallography 56:817).

However, successful crystallization of membrane proteins via such techniques is highly inefficient due to the high tendency of membrane proteins to denature and/or aggregate during crystallization. Furthermore, such methods, being substantially empirical, present the disadvantages of being both time-consuming and of requiring large amounts of pure protein, a requirement which is generally difficult or expensive to fulfill.

One strategy which has been suggested in order to circumvent the disadvantages inherent to such high throughput techniques is to assist the crystallization of molecules which are otherwise difficult or impossible to crystallize by either modifying such molecules so as to facilitate their crystallization, or by crystallizing such molecules in complex with other molecules susceptible to provide an ordered matrix facilitating formation of the basic unit of a crystal lattice.

Protein-modification techniques: One approach attempting to improve membrane protein crystal growth and ordering has employed complexation of a protein of interest with antibody fragments prior to crystallization (Hunte C., 2001. FEBS Lett. 504:126-32; Lange C. & Hunte C., 2002. Proc Natl Acad Sci U S A. 99:2800-5; Ostermeier C. and Michel H., 1997. Curr Opin Struct Biol. 7:697; Ostermeier C. *et al.*, 1997. Proc Natl Acad Sci U S A. 94:10547-53).

Another modification based approach has used fusion of proteins to be

crystallized to large hydrophobic domains derived from heterologous proteins in an attempt to minimize the overall hydrophobicity of proteins of interest (Prive G. *et al.*, 1994. Biol Crystallogr. D50:375).

Yet another approach involves alteration and engineering of crystal unit cell contacts, an example being the crystallization of apoferritin by site-directed mutagenesis of residues involved in the binding of a Co^{2+} atom introduced during the crystallization process (Takeda S. *et al.*, 1995. Proteins, 23:548).

These approaches, however, have the significant drawback that identifying and creating suitable fusion proteins or engineering residues involved in crystal contacts are *ad hoc* and very labor intensive procedures requiring much fine tuning for applicability to any given protein.

Functionalized lipids: Still another approach has employed binding of functionalized lipids to proteins of interest in an attempt to generate crystalline arrays of such proteins. For example, divalent metal ion-chelated lipids or electrostatically charged lipids have been employed to bind proteins via specific surface histidine residues or via complementarily charged residues, respectively. The use of planar layers of such lipids has been employed to generate 2D crystals (Frey W. *et al.*, Proc Nat Acad Sci. USA 1996 93:4937) which can be studied by electron microscopy, but not by X-ray diffraction, thereby yielding limited structural information in terms of dimensionality and in terms of resolution.

A more advanced variant of this approach has utilized lipid nanotubes to generate helical crystals (Wilson-Kubalek, E. *et al.*, Proc. Natl. Acad. Sci. U. S. A. 1998, 95:8040). These crystals, however, can only be used to determine 3D protein structure at low resolution using electron microscopy and thus cannot be employed to solve molecular structure at atomic resolution, as is the case with X-ray crystallography.

Thus, all prior art approaches have failed to provide an adequate solution for efficiently generating X-ray diffraction grade crystals of molecules such as membrane proteins.

There is thus a widely recognized need for and it would be highly

advantageous to have, a method of crystallizing molecules, such as membrane proteins, devoid of the above limitations.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of generating a crystal containing a molecule-of-interest, the method comprising: (a) contacting molecules of the molecule-of-interest with at least one type of heterologous molecular linker being capable of interlinking at least two molecules of the molecule-of-interest to thereby form a crystallizable molecular complex of defined geometry; and (b) subjecting the crystallizable molecular complex to crystallization-inducing conditions, thereby generating the crystal containing the molecule-of-interest.

According to further features in preferred embodiments of the invention described below, the at least one type of heterologous molecular linker is selected such that the crystallizable molecular complex formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

According to still further features in preferred embodiments, the molecule-of-interest is a polypeptide.

According to still further features in preferred embodiments, the polypeptide is a membrane protein.

According to still further features in preferred embodiments, the membrane protein is a G protein coupled receptor.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes a region for specifically binding the molecule-of-interest.

According to still further features in preferred embodiments, the molecule-of-interest is a G protein coupled receptor and the region for specifically binding the molecule-of-interest comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the molecule-of-interest includes a histidine tag and the region for specifically binding the molecule-of-interest comprises a nickel ion or an antibody specific for the histidine tag.

According to still further features in preferred embodiments, the

molecule-of-interest includes core streptavidin and the region for specifically binding the molecule-of-interest comprises a biotin moiety or a Strep-tag.

According to still further features in preferred embodiments, the molecule-of-interest includes a biotin moiety or a Strep-tag and the region for specifically binding the molecule-of-interest comprises core streptavidin.

According to still further features in preferred embodiments, the molecule-of-interest is a G protein coupled receptor and the at least one type of molecular linker comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes at least two non-covalently bound subunits.

According to still further features in preferred embodiments, the at least two non-covalently bound subunits comprise a first subunit comprising a homomultimerizing portion and a metal-binding portion, and a second subunit comprising a portion specifically binding the molecule-of-interest, According to still further features in preferred embodiments, the at least two non-covalently bound subunits comprise a first subunit comprising a homomultimerizing portion and a portion specifically binding the molecule-of-interest, and a second subunit comprising a metal-binding portion, and a portion specifically binding the first subunit.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes a molecule selected from the group consisting of a polycyclic molecule, a polydentate ligand, a macrobicyclic cryptand, a polypeptide and a metal.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker comprises core streptavidin.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker is selected so as to define the spatial positioning and orientation of the at least two molecules within the crystallizable molecular complex, thereby facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes a hydrophilic region, the hydrophilic region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes a conformationally rigid region, the conformationally rigid region being for facilitating crystallization of

the molecule-of-interest.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes a metal-binding moiety capable of specifically binding a metal atom, the metal atom being capable of facilitating crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the metal-binding moiety is a metal binding protein.

According to still further features in preferred embodiments, the metal binding protein is metallothionein.

According to still further features in preferred embodiments, the at least one type of heterologous molecular linker includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable molecular complex and/or of facilitating the interlinking at least two molecules of the molecule-of-interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to still further features in preferred embodiments, the molecule-of-interest includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable molecular complex, and/or of facilitating the interlinking at least two molecules of the molecule-of-interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to still further features in preferred embodiments, the molecule-of-interest includes a metal-binding moiety capable of specifically binding a metal atom, the metal atom being capable of facilitating crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the

metal-binding moiety is a metal binding protein.

According to still further features in preferred embodiments, the metal binding protein is metallothionein.

According to another aspect of the present invention there is provided a method of generating a crystal containing a polypeptide of interest, the method comprising: (a) providing a molecule including the polypeptide of interest and a heterologous multimerization domain being capable of directing the homomultimerization of the polypeptide of interest; (b) subjecting the molecule to homomultimerization-inducing conditions, thereby forming a crystallizable molecular complex; and (c) subjecting the crystallizable molecular complex to crystallization-inducing conditions, thereby generating the crystal containing the polypeptide of interest.

According to further features in preferred embodiments of the invention described below, steps (a) and (b) are effected concomitantly.

According to still further features in preferred embodiments, the heterologous multimerization domain is selected such that the crystallizable molecular complex formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

According to still further features in preferred embodiments, the heterologous multimerization domain includes a hydrophilic region, the hydrophilic region being for facilitating crystallization of the polypeptide of interest.

According to still further features in preferred embodiments, the heterologous multimerization domain includes a conformationally rigid region, the conformationally rigid region being for facilitating crystallization of the polypeptide of interest.

According to still further features in preferred embodiments, the heterologous multimerization domain is selected so as to define the spatial positioning and orientation of polypeptides of the polypeptide of interest within the crystallizable molecular complex, thereby facilitating crystallization of the

polypeptide of interest.

According to still further features in preferred embodiments, the heterologous multimerization domain comprises core streptavidin.

According to still further features in preferred embodiments, the polypeptide of interest is a G protein coupled receptor and the heterologous multimerization domain comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the polypeptide of interest includes a histidine tag and the heterologous

multimerization domain comprises a nickel ion or an antibody specific for the histidine tag.

According to still further features in preferred embodiments, the polypeptide of interest includes core streptavidin and the heterologous multimerization domain comprises a biotin moicty or a Strep-tag.

According to still further features in preferred embodiments, the polypeptide of interest includes a biotin moiety or a Strep-tag and the heterologous multimerization domain comprises core streptavidin.

According to still further features in preferred embodiments, the polypeptide of interest and the heterologous multimerization domain are interlinked via a molecular linker.

According to still further features in preferred embodiments, at least one of the heterologous multimerization domain and the molecular linker include a hydrophilic region, the hydrophilic region being for facilitating crystallization of the polypeptide of interest.

According to still further features in preferred embodiments, at least one of the heterologous multimerization domain and the molecular linker include a conformationally rigid region, the conformationally rigid region being for facilitating crystallization of the polypeptide of interest.

According to still further features in preferred embodiments, at least one of the heterologous multimerization domain and the molecular linker is selected so as to define the spatial positioning and orientation of polypeptides of the polypeptide of interest within the crystallizable molecular complex, thereby facilitating crystallization of the polypeptide of interest.

According to still further features in preferred embodiments, the at least one molecular linker includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable molecular complex, and/or of facilitating the homomultimerization of the polypeptide of interest.

According to still further features in preferred embodiments, the region

being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to still further features in preferred embodiments, the polypeptide of interest includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable molecular complex, and/or of facilitating the homomultimerization of the polypeptide of interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to still further features in preferred embodiments, the molecule includes a metal-binding moicty capable of specifically binding a metal atom, the metal atom being capable of facilitating crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the metal-binding moicty is a metal binding protein.

According to still further features in preferred embodiments, the metal binding protein is metallothionein.

According to still further features in preferred embodiments, the polypeptide of interest is a membrane protein.

According to still further features in preferred embodiments, the membrane protein is a G protein coupled receptor.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the polypeptide of interest includes a metal-binding moiety capable of specifically binding a metal atom, the metal atom being capable of facilitating crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the metal

binding moiety is metallothionein.

According to yet another aspect of the present invention there is provided a composition-of-matter comprising at least two molecules of a molecule-of-interest interlinked via a heterologous molecular linker, wherein the heterologous molecular linker is selected so as to define the relative spatial positioning and orientation of the at least two molecules within the composition-of-matter, thereby facilitating formation of a crystal therefrom under crystallization-inducing conditions.

According to further features in preferred embodiments of the invention described below, the molecule-of-interest is a polypeptide.

According to still further features in preferred embodiments, the polypeptide is a membrane protein.

According to still further features in preferred embodiments, the membrane protein is a G protein coupled receptor.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the heterologous molecular linker includes at least one region capable of specifically binding the molecule-of-interest.

According to still further features in preferred embodiments, the molecule-of-interest is a G protein coupled receptor and the at least one region capable of specifically binding the molecule-of-interest is a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the heterologous molecular linker includes a molecule selected from the group consisting of a polycyclic molecule, a polydentate ligand, a macrobicyclic cryptand, a polypeptide and a metal.

According to still further features in preferred embodiments, the molecule-of-interest is a G protein coupled receptor and the heterologous molecular linker comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

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According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the heterologous molecular linker comprises core streptavidin.

According to still further features in preferred embodiments, the heterologous molecular linker includes at least two non-covalently bound subunits.

According to still further features in preferred embodiments, the heterologous molecular linker includes a hydrophilic region, the hydrophilic region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the heterologous molecular linker includes a conformationally rigid region, the conformationally rigid region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the heterologous molecular linker is selected such that the composition-of-matter is capable of generating a crystal selected from the group consisting of a 2D crystal,

a helical crystal and a 3D crystal.

According to still further features in preferred embodiments, the heterologous molecular linker includes a metal-binding moiety capable of specifically binding a metal atom, the metal atom being capable of facilitating crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the metal-binding moiety is a metal-binding protein.

According to still further features in preferred embodiments, the metal binding protein is metallothionein.

According to still further features in preferred embodiments, the heterologous molecular linker includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable composition-of-matter, and/or of facilitating the interlinking of the at least two molecules of a molecule-of-interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to still further features in preferred embodiments, the molecule-of-interest includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the composition-of-matter, and/or of facilitating the interlinking of the at least two molecules of a molecule-of-interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to still further features in preferred embodiments, the molecule-of-interest includes a metal-binding moiety capable of specifically binding a metal atom, the metal atom being capable of facilitating crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the

metal-binding moiety is a metal binding protein.

According to still further features in preferred embodiments, the metal-binding protein is metallothionein.

According to still another aspect of the present invention there is provided a nucleic acid construct comprising a polynucleotide segment encoding a chimeric polypeptide including: (a) a first polypeptide region being capable of specifically binding a molecule-of-interest; and (b) a second polypeptide region being capable of specifically binding a metal atom.

According to further features in preferred embodiments of the invention described below, the molecule-of-interest is a G protein coupled receptor and the chimeric polypeptide comprises SEQ ID NO: 5 or SEQ ID NO: 6.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the molecule-of-interest is a G protein coupled receptor and the first polypeptide region comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation

at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the molecule-of-interest is a polypeptide.

According to still further features in preferred embodiments, the polypeptide is a membrane protein.

According to still further features in preferred embodiments, the membrane protein is a G protein coupled receptor.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the second polypeptide region is metallothionein.

According to still further features in preferred embodiments, the chimeric polypeptide is selected such that when combined with molecules of the molecule-of-interest under suitable conditions, the chimeric polypeptide and the molecules form a crystallizable molecular complex which is capable of forming a crystal containing the molecule-of-interest when subjected to crystallization-inducing conditions.

According to still further features in preferred embodiments, the chimeric polypeptide is selected such that when combined with molecules of the molecule-of-interest and the metal atom under suitable conditions, the chimeric

polypeptide and the molecules form a crystallizable molecular complex which is capable of forming a crystal containing the molecule-of-interest when subjected to crystallization-inducing conditions.

According to still further features in preferred embodiments, the metal atom facilitates crystallographic analysis of the crystal.

According to still further features in preferred embodiments, the chimeric polypeptide includes a hydrophilic region, the hydrophilic region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the chimeric polypeptide includes a conformationally rigid region, the conformationally rigid region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the chimeric polypeptide is selected so as to define the spatial positioning and orientation of the molecule-of-interest within the crystallizable molecular complex, thereby facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the chimeric polypeptide is selected such that the crystallizable molecular complex formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

According to still further features in preferred embodiments, the chimeric polypeptide further includes a polypeptide region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable molecular complex, and/or of facilitating the binding of a molecule-of-interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

According to a further aspect of the present invention there is provided a nucleic acid construct comprising a polynucleotide segment encoding a chimeric polypeptide including: (a) a first polypeptide region being capable of specifically

binding a molecule-of-interest; (b) a second polypeptide region being capable of homomultimerization into a complex of defined geometry; and (c) a third polypeptide region being capable of specifically binding a metal atom.

According to further features in preferred embodiments of the invention described below, the molecule-of-interest is a G protein coupled receptor and the first polypeptide region is selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the second polypeptide region comprises core streptavidin.

According to still further features in preferred embodiments, the

molecule-of-interest is a G protein coupled receptor and the chimeric polypeptide comprises SEQ ID NO: 5 or SEQ ID NO: 6.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the third polypeptide region comprises metallothionein.

According to still further features in preferred embodiments, the molecule-of-interest is a polypeptide.

According to still further features in preferred embodiments, the polypeptide is a membrane protein.

According to still further features in preferred embodiments, the membrane protein is a G protein coupled receptor.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the chimeric polypeptide is selected such that when combined with molecules of the molecule-of-interest, the chimeric polypeptide and the molecules form a crystallizable molecular complex of defined geometry which is capable of forming a crystal containing the molecule-of-interest when subjected to crystallization-inducing conditions.

According to still further features in preferred embodiments, the chimeric polypeptide includes a hydrophilic region, the hydrophilic region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the chimeric polypeptide includes a conformationally rigid region, the conformationally rigid region being for facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the chimeric polypeptide is selected so as to define the spatial positioning and orientation of molecules of the molecule-of-interest within the crystallizable molecular complex, thereby facilitating crystallization of the molecule-of-interest.

According to still further features in preferred embodiments, the chimeric polypeptide is selected such that the crystallizable molecular complex of defined geometry formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

According to still further features in preferred embodiments, the metal atom facilitates crystallographic analysis of the molecule-of-interest contained in the crystal.

According to still further features in preferred embodiments, the chimeric polypeptide further includes a polypeptide region being capable of functioning as a purification tag, the purification tag being capable of facilitating purification of the crystallizable molecular complex, and/or of facilitating the binding of a molecule-of-interest.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, and core streptavidin.

According to a yet a further aspect of the present invention there is provided a method of purifying a G protein coupled receptor from a sample containing the G protein coupled receptor, the method comprising subjecting the sample to affinity chromatography using an affinity ligand selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, a molecule defined by SEQ ID NO: 3, and a molecule defined by SEQ ID NO: 4, thereby purifying the G protein coupled receptor.

According to further features in preferred embodiments of the invention described below, the at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

According to still further features in preferred embodiments, the at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of the arrestin molecule.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

According to still further features in preferred embodiments, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

According to still further features in preferred embodiments, the G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

According to still further features in preferred embodiments, the class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

According to still further features in preferred embodiments, the affinity ligand includes a region being capable of functioning as a purification tag, the purification tag being capable of facilitating attachment of the affinity ligand to an affinity chromatography matrix.

According to still further features in preferred embodiments, the region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be

the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1a is a diagram depicting the general configuration of a non-polypeptidic molecular linker which can be used for multimerization of a molecule-of-interest according to the teachings of the present invention. MS: molecular scaffold, M: metal atom; L: linking chain containing 1-3 carbon or oxygen atoms (shown in Figure 1b); G = [$-CO_2$], [-CONH], [-O], [-OCO] or [-NHCO]; L' = linking chain of 1-10 atoms containing carbon or oxygen atoms, such as [$(CH_2CH_2O)_2$-O-CH_2CH_2-] or [$-(CH_2)_4$-]; SBD = specific binding domain, such as [$-N^+(CH_3)_3$] or [$-CO(CF_3)$], or a polypeptide such as biotin.

FIG. 1b is a diagram depicting a linking chain containing 1–3 carbon or oxygen atoms comprised in the non-polypeptidic molecular linker described in Figure 1a. G' = [CO_2H], [OH] or [NH_2].

FIGs. 2a-b are diagrams depicting porphyrin-based molecular linkers which can be used according to the teachings of the present invention for multimerization of two (Figure 2a) or four (Figure 2b) molecules of interest. X = [L-G-L'-SBD], as defined in Figure 1a; R = H, (sub)-phenyl or [L-G-L'-SBD], as defined in Figure 1a, M = metal atom.

FIG. 3 is a diagram depicting a hydroxime-based molecular linker which can be used according to the teachings of the present invention for multimerization of two molecules of interest. X = [L-G-L'-SBD], R' = H, (sub)-phenyl or [L-G-L'-SBD], as defined in Figure 1a; R' = H or methyl group; M = metal atom.

FIGs. 4a-b are schematic diagrams depicting synthesis of the porphyrin molecular linkers of Figures 2a-b which can be used for multimerization of four

(Figure 4a) or two (Figure 4b) molecules of interest. HY = a strong acid; MZ_2 = a transition or heavy metal salt; Oxid = an oxidant, such as DDQ or O_2.

FIG. 5 is a schematic diagram depicting synthesis of the hydroxime-based molecular linker of Figure 3. MZ_2 = a transition or heavy metal salt.

FIG. 6a is a schematic diagram depicting linkage of a biotinylated moiety to porphyrin-based molecular linkers such as those depicted in Figures 2a-b.

FIG. 6b is a schematic diagram depicting linkage of a trimethylammonium moiety to hydroxime-based molecular linkers such as the one depicted in Figure 3. MZ_2 = a transition or heavy metal salt.

FIGs. 7a-b are schematic diagrams depicting polynucleotide constructs for purification of molecules of interest. Figure 7a is a diagram depicting a construct encoding a chimeric polypeptide containing a single-chain Fv (scFv) segment fused to a core streptavidin and purification tag segments. Figure 7b is a diagram depicting a construct encoding a chimeric polypeptide containing a Strep-tag (Stag) segment fused to a metal atom binding polypeptide (MBP) segment fused in turn to a purification tag segment. The relative positions of the Strep-tag and metal atom binding polypeptide can also be inverted. NH_2–amino-terminus; leader–leader sequence or signal peptide for expression in eukaryotic or prokaryotic cells; V_H and V_L–antibody variable heavy and light chains, respectively.

FIG. 8 is a diagram depicting the conformation of a core-streptavidin tetramer used in the molecular linkers of the present invention indicating the N-terminal fusion sites thereof for attachment of moieties capable of specifically binding a molecule-of-interest, such as a single-chain Fv, and the binding site for attachment of a Strep-tag or a biotin moiety.

FIGs. 9a-b are sequence diagrams depicting the amino acid residue sequence of portions of human beta-arrestin-1a suitable for binding different classes of GPCRs with high affinity and specificity independently of the phosphorylation-activation state thereof. Figure 9a depicts a polypeptide composed of amino acid residues 11–190 of human beta-arrestin-1a with

mutation R169E. Figure 9b depicts a polypeptide composed of amino acid residues 11–370 of human beta-arrestin-1a with mutation R169E. In both polypeptides, mutation R169E conferring the capacity to bind GPCRs independently of the phosphorylation-activation state thereof, and the wild type serine residue at position 86 conferring the capacity to bind multiple types of GPCRs are indicated (bold underlined).

FIGs. 10a-b are sequence diagrams depicting the amino acid residue sequence of molecular linkers for crystallization of different classes of GPCRs independently of the phosphorylation-activation state thereof. Figure 10a depicts a linker composed of a chimeric protein consisting of the N- to C-terminal segments; T7 tag (N-terminal italics), core streptavidin (uppercase), the peptide linker GSAA (SEQ ID NO: 1; internal italics), and amino acid residues 11–190 of human beta-arrestin-1a (lowercase) with mutation R169E. Figure 10b depicts a linker composed of a chimeric protein consisting of the N- to C-terminal segments; T7 tag (N-terminal italics), core streptavidin (uppercase), the peptide linker GSAA (SEQ ID NO: 1; internal italics), and amino acid residues 11–370 of human beta-arrestin-1a (lowercase) with mutation R169E. In the arrestin derived segment of both molecular linkers, mutation R169E conferring the capacity to bind GPCRs independently of the phosphorylation-activation state thereof, and the wild type serine residue at position 86 conferring the capacity to bind multiple types of GPCRs are indicated (bold underlined).

FIG. 11 is a chemical structure diagram depicting a porphyrin-NTA-Ni^{2+} molecular linker used for crystallization of histidine-tagged proteins.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and compositions which can be used for generating crystals containing a molecule-of-interest, and of methods of purifying G protein coupled receptors (GPCRs). Specifically, the present invention can be used to generate crystals of membrane proteins which can be used to determine the three-dimensional (3D) atomic structure thereof, and to

purify GPCRs using arrestin derived polypeptides as affinity ligands of GPCRs.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Various methods of assisting the crystallization of molecules such as polypeptides and of facilitating their crystallographic analysis have been described in the prior art.

Techniques involving protein modifications, such as those based on fusion of the polypeptide of interest to a large heterologous hydrophobic polypeptide domain, alteration and engineering of crystal unit cell contacts or complexation of a protein of interest with antibody fragments are typically dedicated, labor intensive and require much fine tuning. In addition, methods relying on artificial functionalized lipid scaffolds are only useful for the creation of planar 2D crystals which can be studied by electron microscopy, but not by X-ray diffraction, or are useful for generation of helical crystals which do not permit high resolution 3D structural analysis.

Thus, prior art approaches for assisting or facilitating crystallization of molecules-of-interest have failed to provide adequate solutions for the controlled 3D crystallization of molecules such as polypeptides, while allowing subsequent determination of their 3D atomic structure.

In sharp contrast to prior art techniques, the methods of the present invention enable the generation of readily crystallizable molecular complexes incorporating molecules of a molecule-of-interest, such as a membrane protein. In addition, the present invention also enables purification of the

molecule-of-interest, thereby greatly facilitating crystallographic analysis thereof.

Thus, according to the present invention, there is provided a method of generating a 2D, or preferably a 3D, crystal containing a molecule-of-interest.

According to one embodiment of the method of the present invention, crystallization of a molecule-of-interest is effected by contacting molecules of the molecule-of-interest with at least one type of linker. The linker is selected so as to be capable of interlinking at least two molecules of the molecule-of-interest to thereby form a crystallizable molecular complex of defined geometry (defined spatial orientation). As is further described hereinunder, the linker can be composed of a single molecule or a complex including a plurality of molecules, depending on the application and purpose.

Following linker–molecule-of-interest binding, the molecular complex formed is subjected to crystallization-inducing conditions, such as those described in Example 6 of the Examples section, thereby generating the crystal containing the molecule-of-interest.

As mentioned hereinabove, both single molecule and multi-molecule linker configurations can be used by the present invention.

A single-molecule linker can include binding regions covalently attached to a core, while a multi-molecule linker (linker complex) can include binding regions non-covalently associated with a core unit, and/or may include a core unit composed of non-covalently associated subunits. In any case, the linker is designed and configured such that when complexed with molecules of a molecule-of-interest, the linker directs the spatial orientation of the molecules of the molecule-of-interest so as to form a molecular complex of pre-defined geometry, thereby facilitating crystallization of the molecule-of-interest when the molecular complex is subjected to crystallization inducing conditions. The following Examples section describes specific examples of single-molecule and multi-molecule type linkers, as further detailed hereinbelow.

As used herein, a "core" of a linker refers to a portion of the linker functioning as the basic molecule-of-interest multimerization scaffold of the

linker.

Regardless of core configuration, minimizing core size may be advantageous depending on the application and purpose. Cores of minimal size may be generally advantageous since this may minimize the size of the linker, which in turn serves to maximize tightness of packing of the molecular complex. This minimizes conformational disorder in the molecular complex, thus ensuring optimal ordering of crystals. As a further advantage, minimizing core size may make the linker easier and/or cheaper to produce and purify.

Single molecule linkers, being composed of covalently connected atoms, are highly stable and rigid and can be advantageously used to generate molecular complexes having minimized conformational disorder, for example, relative to linker complexes. Thus, single molecule linkers can be used to generate optimally ordered crystals, and may be more conveniently, cheaply, and/or easily produced relative to linker complexes.

Linker complexes may advantageously comprise homomultimerized proteins, such as, for example, fusion proteins comprising a homomultimerizing domain and a polypeptide or polypeptides, such as a binding domain and/or a purification tag, being capable of facilitating crystallization and/or 3D structure determination of a molecular complex, as further described hereinbelow. The use of linker complexes comprising such homomultimerized fusion proteins may be advantageously employed to obviate the need to separately express the polypeptide components of such fusion proteins, as well as the need to subject such components to conditions facilitating their association, thereby greatly facilitating generation of the linker complex, generation of the molecular complex, and/or crystallization of a molecule-of-interest.

The linkers of the present invention include one or preferably several binding domains for specifically binding the molecule-of-interest. Such binding domains can be synthesized as part of the linker or as distinct molecules which can be non-covalently associated with a core molecule to form the linker (linker complex).

Non-covalent association of binding domains to linkers can be advantageously used to enable the linkers of the present invention to be modular, such that one type of molecular linker core can be used to associate essentially any desired binding domain according to the target molecule to be complexed and crystallized.

Binding domains which bind molecules of a molecule-of-interest covalently or binding domains which bind molecules of a molecule-of-interest non-covalently can be used, depending on the application and purpose.

Binding domains which bind a molecule-of-interest non-covalently can be advantageously used to bind a molecule-of-interest without the need to resort to chemical synthesis techniques required for covalently coupling molecules. In the case of a biomolecular molecule-of-interest, the availability of highly specific ligands, such as, for example, antibodies, provides a pool of molecules useable as highly efficient binding domains.

Binding domains which bind a molecule-of-interest covalently can be advantageously used to bind a molecule-of-interest with great stability, thereby minimizing conformational disorder in crystals generated therewith, relative, for example, to binding domains which bind a molecule-of-interest non-covalently.

Preferably, single molecule linkers are porphyrin based. Porphyrin based linkers can be advantageously used to multimerize molecules of a molecule-of-interest with great stability and rigidity, as described in Example 1 of the following Examples section.

Multimerized streptavidin or streptavidin derived molecules may be advantageously utilized as the core of a molecular linker.

Preferably, the streptavidin molecule or streptavidin derived molecule is a core streptavidin. Suitable core streptavidins may comprise, for example, amino acid residues 13–133, 13–131 or 16–131 of native streptavidin.

The use of core streptavidin as the core of molecular linkers is advantageous since core streptavidin homomultimerizes into a particularly tightly packed tetramer, for example relative to native streptavidin tetramer. As a

further advantage, core streptavidin tetramers display enhanced stability under denaturing conditions, and their biotin binding sites appear to be more accessible relative to native streptavidin tetramer. Extensive literature exists for the expression, purification and uses of streptavidin or streptavidin derived molecules (Wu SC. *et al.*, 2002. Protein Expression and Purification 24:348-356; Gallizia A. *et al.*, 1998. Protein Expression and Purification 14:192-196), fusion proteins comprising streptavidin or streptavidin derived molecules (Sano T. & Cantor CR. 2000. Methods Enzymol. 326:305-11), and modified streptavidin or streptavidin derived molecules (see, for example: Sano T. *et al.*, 1993. Journal of Biological Chemistry 270:28204-28209), including for streptavidin or streptavidin derived molecules whose gene sequence has been optimized for expression in *E. coli* (Thompson LD. & Weber PC., 1993. Gene 136:243-6).

Fusion proteins comprising core streptavidins may be optimal when comprising an N-terminal core streptavidin segment and/or when produced as inclusion bodies. This may optimize correct folding and/or maximize the number of free biotin binding sites.

Molecular linkers including multimerized fusion proteins comprising core streptavidin and a polypeptidic binding domain, such as a single chain antibody Fv or a biological ligand of the molecule-of-interest, can be conveniently used to efficiently crystallize a molecule-of-interest.

Synthesis of chimeric polypeptides comprising core streptavidin and a single chain Fv can be effected by cloning nucleic acid sequences encoding the single chain Fv in an expression vector configured to express an in-frame chimeric polypeptide comprising core streptavidin, and the single chain Fv in a suitable host such as *E. coli* following transformation thereof using standard recombinant polypeptide expression technology.

Detailed protocols for the synthesis of streptavidin-single chain Fv fusion proteins can be found in the literature of the art (for example refer to Cloutier SM. *et al.*, 2000. Molecular Immunology 37:1067-1077; Dubel S. *et al.*, 1995. J Immunol Methods 178:201; Huston JS. *et al.*, 1991. Methods in Enzymology

203:46; Kipriyanov SM. *et al.*, 1995. Hum Antibodies Hybridomas 6:93; Kipriyanov SM. *et al.*, 1996. Protein Engineering 9:203; Pearce LA. *et al.*, 1997. Biochem Molec Biol Intl 42:1179-1188).

As is shown in Examples 7 and 9 of the Examples section which follows, core streptavidin based molecular linkers can be used to crystallize a molecule-of-interest.

Suitable binding domains which bind a molecule-of-interest non-covalently include but are not limited to, polypeptides derived from antibodies, such as, for example, single-chain Fv fragments, as described in Example 7 of the Examples section, T cell receptors, MHC-peptide complexes, biological ligands of the molecule-of-interest, and affinity-selected peptides, such as phage-display selected peptides.

As described in Example 7 of the Examples section, single-chain Fv fragments can be advantageously used to specifically bind and crystallize a molecule-of-interest.

In general, synthesis a single chain Fv molecule specific for a molecule-of-interest comprises producing and screening hybridoma cell lines secreting an antibody specific for the molecule-of-interest via standard hybridoma production techniques, and using RT-PCR to clone cDNA sequences encoding the variable light and variable heavy chains of the antibody. Ample guidance regarding production of single chain Fv's and fusion proteins comprising single chain Fv's is available in the literature of the art.

Suitable binding domains which bind a molecule-of-interest covalently include various chemical groups such as, for example, $[-N^{+}(CH_3)_3]$ and $[-CO(CF_3)]$ (trifluorocarbonyl), as described in Example 1 of the Examples section, and N-(5-amino-1-carboxypentyl)imino-diacetic acid (NTA), as described in Example 11 of the following Examples section. Covalent coupling of a molecule-of-interest to a linker can be effected using standard chemical techniques for which guidance is broadly available in the literature of the art. For example, a trifluorocarbonyl group can be bound to the amino end, as well as to

amino acid residues having free -OH, -SH, -NH2 groups of a polypeptidic molecule-of-interest, via a reaction of these groups with a compound such as HO-C(=O)-CF_3, under appropriate conditions.

It will be appreciated that other than as described hereinabove, linker universality can also be achieved by modifying the molecule to be crystallized to include specific binding moieties recognized by a single and universal linker, for example as described in Example 8 of the Examples section below. In the case of a polypeptidic molecule-of-interest, the molecule-of-interest can be expressed as part of a chimeric polypeptide including the binding moiety. Alternatively, the moiety is chemically attached to the molecule-of-interest. In any case, the binding moiety is preferably selected such that it readily associates with the linker while not substantially modifying the structure of the molecule to be crystallized.

Examples of binding domains of such universal linkers include biotin, as described in Examples 2 and 4 of the Examples section, an antibody-derived molecule, such as an anti purification tag single-chain Fv fragment, as described in Example 7 of the Examples section, a nickel ion, as described in Example 11 of the Examples section below, or essentially any specific ligand of a purification tag.

Examples of moieties which can be used to modify a molecule-of-interest such that it may be bound by universal linkers comprising specific ligands of purification tags include various purification tags.

As used herein, the term "purification tags" encompasses affinity tags.

Examples of purification tags include epitope tags, histidine tags, Strep-tags, single-chain Fv molecules, core streptavidin, streptavidin, and biotin.

Guidance regarding tagging molecules with histidine tags, and uses of such molecules is available in the literature of the art (for example, refer to: Sheibani N. 1999. Prep Biochem Biotechnol. 29:77).

Guidance regarding tagging molecules with Strep-tags, and uses of such molecules is available in the literature of the art (for example, refer to: Schmidt,

TGM. and Skerra, A. Protein Eng. 1993, 6:109; Skerra A. & Schmidt TGM., 1999. Biomolecular Engineering 16:79-86).

Epitope tags can be comprised in a molecule-of-interest to enable complexation with linkers comprising single-chain Fv domains specific for such epitope tags.

Examples of epitope tags include an 11-mer *Herpes simplex* virus glycoprotein D peptide, and an 11-mer N-terminal bacteriophage t7 peptide, being commercially known as HSVTag and T7 Tag, respectively (Novagen, Madison, WI, USA), and 10- or 9-amino acid c-myc or *Hemophilus influenza* hemagglutinin (HA) peptides, which are recognized by the variable regions of monoclonal antibodies 9E10 and 12Ca5, respectively.

Examples of moieties which can be used to modify molecules of interest such that these may be bound by a linker comprising biotin include streptavidin, core streptavidin and anti biotin single-chain antibody Fv.

Examples of moieties which can be used to modify molecules of interest such that these may be bound by a linker comprising streptavidin include Strep-tags, as described in Example 8 of the Examples section, or biotin.

Examples of moieties which can be used to modify molecules of interest such that these may be bound by a linker comprising a metal atom include, but are not limited to, histidine tags.

In the case of polypeptidic molecules-of-interest, polypeptide tags, such as, for example, histidine tags or Strep-tags, are particularly convenient since the molecule-of-interest and the tag can be co-expressed as a chimeric protein.

As mentioned hereinabove, the linkers of the present invention facilitate crystallization of molecules of interest by enabling the generation of a molecule-linker complex in which bound molecules are positioned in a defined spatial orientation. To allow such spatial positioning, the linker is selected of a size and geometric configuration which is capable of restricting the bound molecules to a predetermined orientation thus greatly facilitating 3D crystal formation.

Linker size and geometric configuration selection are also influenced by the need to maximize molecule-molecule interactions during or following complex formation. Such molecule-molecule interactions enhance the stability of the complex formed and thus further facilitate crystal formation therefrom.

It will be appreciated that linker length and spatial configuration selection is effected in accordance with the molecule to be crystallized. Such selection may be advantageously facilitated using computerized 3D modeling of the assembled crystallization complex. Such computerized 3D modeling is routinely effected by the ordinarily skilled practitioner using software available via the Internet/World Wide Web. Suitable software applications which may be used to generate 3D structure models of molecules include RIBBONS (Carson, M. (1997) Methods in Enzymology 277: 25), O (Jones, TA. *et al.* (1991) Acta Crystallogr A47:110), DINO (DINO: Visualizing Structural Biology (2001) http://www.dino3d.org); and QUANTA, CHARMM, INSIGHT, SYBYL, MACROMODE, ICM, MOLMOL, RASMOL and GRASP (reviewed in Kraulis, J. (1991) Appl Crystallogr. 24:946).

For example, in the case of membrane proteins, a core streptavidin-single-chain Fv linker (Example 7) can be used to tetramerize a membrane protein to form a non-planar geometric configuration. Such a non-planar geometric configuration would prevent the membrane protein from forming disordered aggregates or 2D crystals and would thus enable the generation of 3D crystals therefrom.

In the case of molecules which lack sufficient conformational rigidity, the linkers employed are designed so as to provide rigidity to bound molecules thereby further facilitating crystallization thereof.

Such conformational rigidity can be obtained by utilizing linkers having cores based on polydentate ligands, including, but not limited to, polydentate ligands, such as porphyrin, or macrobicyclic cryptands, such as hydroxime, as described in Examples 1-5 and 11 of the Examples section which follows. As described hereinabove, core streptavidin tetramer can be used to generate a

suitably conformationally rigid linker.

In addition to the above described features, the linkers employed by the present invention can also include several additional features.

According to another preferred embodiment of the present invention, the linkers include a hydrophilic domain such that complexes formed thereby are sufficiently hydrophilic so as to facilitate crystallization of molecules of interest which are substantially hydrophobic.

Examples of such "hydrophilic" linkers include, for example, linkers comprising core streptavidin or single-chain Fv, as described in Example 7 of the Examples section, linkers comprising non-polypeptidic hydrophilic molecules such as, for example, trimethylammonium, as described in Example 5 of the Examples section, or linkers comprising N-(5-amino-1-carboxypentyl)imino-diacetic acid (NTA) groups, as described in Example 11 of the Examples section below.

According to another preferred embodiment of the present invention, the linkers include a purification tag, for example, as described hereinabove. Such a purification tag can be advantageously used for purification of the linker and/or of the molecular complex.

Purification of a molecule-of-interest is a critical and limiting step in the crystallization of a molecule-of-interest, such as a polypeptidic molecule-of-interest and, as such, methods for improving such purification can serve to thereby greatly facilitate the crystallization of such molecules of interest. The same considerations may be applicable to purification of the linkers, such as the polypeptide-based linkers of the present invention.

Examples of suitable purification tags include, for example, the epitope tags to which specific antibodies exist which are listed and described hereinabove, a Strep-tag and a histidine tag, as described in Example 7 of the Examples section. Purification of a molecule containing a histidine tag is routinely performed by those well-versed in the art, using nickel-based automatic affinity column purification techniques. Purification of a molecule containing a

Strep-tag can be effected using standardized techniques, for example, as described hereinabove.

The method of the present invention can be used to crystallize any known type of molecules including inorganic and organic molecules.

Examples of organic molecules include, but are not limited to, polypeptides such as membrane proteins, receptors, enzymes, antibodies and prions, as well as nucleic acids, carbohydrates, hormones, polycyclic molecules and lipids.

The present invention can be advantageously used to crystallize a GPCR.

Preferably, the present invention is used to crystallize a GPCR such as rhodopsin or a class A GPCR.

Preferably, the present invention is used to crystallize a class A GPCR such as m2 muscarinic cholinergic receptor.

Guidance regarding families, types or classes of GPCRs, including mutant GPCRs, is widely available in the literature of the art (see, for example: Edvardsen O. *et al.*, 2002. Nucleic Acids Res. 30:361; Attwood TK. *et al.*, 2002. Protein Eng. 15(1):7)

Crystallization of GPCRs is preferably effected using molecular linkers comprising as a binding domain a GPCR-binding domain of an arrestin molecule.

Types of arrestins which can be used according to the method of the present invention include, but are not limited to, beta-arrestin-1a (Lohse MJ. *et al.*, 1990. Science 248:1547-1550; Parruti,G. *et al.*, 1993. J Biol Chem. 268:9753-9761; Calabrese G. *et al.*, 1994. Genomics 24:169-171; Lefkowitz RJ., 1998. J Biol Chem. 273:18677-18680; Luttrell LM. *et al.*, 1999. Science 283:655-661), arrestin-C (Craft CM. *et al.*, 1994. J Biol Chem. 269:4613-4619), S-arrestin (Yamaki K. *et al.*, 1990. J Biol Chem. 265:20757-20762; Calabrese G. *et al.*, 1994. Genomics 23:286-288; Yamamoto S. *et al.*, 1997. Nat Genet. 15:175-178; Sippel KC. *et al.*, 1998. Invest Ophthalmol Vis Sci. 39:665-670), arrestin 3 (Murakami A. *et al.*, 1993. FEBS Lett. 334:203-209; Craft CM. *et al.*, 1994. J Biol Chem. 269:4613-4619; Sakuma H. *et al.*, 1996. FEBS Lett. 382:105-110), beta-arrestin-2 (Rapoport B. *et al.*, 1992. Mol Cell Endocrinol. 84:R39-R43; Attramadal H. *et al.*, 1992. J Biol Chem. 267:17882-17890; Calabrese G. *et al.*, 1994. Genomics 23:286-288; Lefkowitz RJ., 1998. J Biol Chem. 273:18677-18680), and beta-arrestin-1b (Lohse MJ. *et al.*, 1990. Science 248:1547-1550; Parruti G. *et al.*, 1993. J Biol Chem. 268:9753-9761; Calabrese G. *et al.*, 1994. Genomics 24:169-171; Lefkowitz RJ., 1998. J Biol Chem. 273:18677-18680; Luttrell LM. *et al.*, 1999. Science 283:655-661). Ample guidance regarding the location of G protein coupled receptor binding domains of arrestins is provided in the aforementioned references and in the Examples section which follows.

Preferably, the arrestin molecule is beta-arrestin-1a.

Regardless of the type of arrestin used, the GPCR binding domain is preferably homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

Preferably, the G protein coupled receptor-binding domain has a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, or more preferably both.

Preferably, the mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a threonine residue or more preferably to a serine residue.

Preferably, the mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a an asparagine residue or more preferably to a glutamic acid residue.

Guidance regarding identification of amino acid residue positions in various arrestins corresponding to amino acid residue positions in bovine visual arrestin can be found in the literature of the art (see, for example: Han M. *et al.*, 2001. Structure (Camb) 9:869-80; Hirsch JA. *et al.*, 1999. Cell 97:257-69).

In general, corresponding amino acid residue positions between any pair

of related proteins, such as a pair of arrestins, may be computationally determined using software tools suitable for aligning proteins, such as alignment software of the NCBI available on the World Wide Web/Internet.

As is described in Example 9 of the following Examples section, GPCR-binding domains of arrestins having a serine residue at an amino acid residue position corresponding to position 90, or a glutamic acid residue an amino acid residue position corresponding to position 175 in bovine visual arrestin can, respectively, be advantageously used to bind different types of GPCRs or to bind GPCR independently of its activation-phosphorylation state, respectively.

Preferably, the GPCR binding domain corresponds to the amino acid sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 4. As shown in Example 9 of the Examples section below molecular linkers comprising SEQ ID NO: 3 or SEQ ID NO: 4 can be used to specifically bind various types of GPCRs with high affinity and specificity regardless of the activation state of such GPCRs.

Crystallization of the linker-molecule complex can be effected via any of the standard means described in the literature, including, for example, microbatch, vapor diffusion or dialysis (Bergfors, T.M., *Protein crystallization*. IUL Biotechnology Series. 1999, La Jolla, CA: International University Line). In such methods, the appropriate amount of linker is added to a monodisperse solution of the molecule-of-interest and the solution is then employed in any of the methods mentioned above. For example, the optimal amount of reagents, such as linker subunits, to be added for facilitating crystallization can be determined by dynamic light scattering so as to ensure monodispersity of the crystallizable molecular complex and to measure the second virial coefficient, which can be employed as a diagnostic indicator for the tendency of the molecular species in solution to crystallize (George, A., *et al.*, *Macromolecular Crystallography*, Pt a. 1997. p. 100).

To facilitate X-ray crystallographic determination of the structure of a crystallized molecule-of-interest, the molecular complexes of the present

invention can further include at least one metal atom associated therewith. Such a metal atom can be used to generate initial phases for X-ray diffraction crystallography, via methodologies such as multiple anomalous diffraction (MAD) (Hendrickson WA., Science 1991, 254:51), thereby facilitating solution, for example, of the 3D atomic structure of the crystallized molecule.

Alternately, X-ray crystallographic structure determination of the molecule-of-interest may be facilitated by association of a metal atom with the molecule-of-interest.

Examples of such metal atoms include, for example, iron, cobalt, nickel, cadmium, platinum and zinc.

To be capable of associating with a metal atom, the linkers of the present invention may include polydentate ligands, such as porphyrin, and macrobicyclic cryptands, such as hydroxime, as described in Example 1 of the Examples section.

Alternately, to be capable of associating with a metal atom, the linkers of the present invention or a molecule-of-interest may include, for example, a metal binding protein, such as metallothionein, desulforedoxin, rubredoxin, colicin or rubrerythrin.

Preferably, the metal binding protein is metallothioncin.

Conjugation of a metal binding protein with a polypeptidic linker or molecule-of-interest can be conveniently effected by co-expressing the metal binding protein with the linker or the molecule-of-interest as a fusion protein.

For example, metallothionein-streptavidin fusion proteins may be generated as previously described (Sano T. *et al.*, 1999. Proc Natl Acad Sci U S A. 89:1534-8).

As shown in Example 9 of the Examples section below, a molecular linker comprising metallothionein can be used to generate a highly ordered crystal of a membrane protein, which crystal comprising a metal atom useful for determining initial phases for structural analysis of such a membrane protein.

It will be understood by one versed in the art that metal atoms facilitating

crystallographic analysis, as described in the present invention, include the ionized forms of such metal atoms, such as, for example, Pt^{2+}, Ni^{2+}, Cu^{2+} or Co^{2+}.

It will be appreciated that such a metal atom can also serve as a nucleating core around which linker arms can associate into a linker complex as described hereinabove.

Thus, the present invention enables crystallization of any molecule-of-interest and, in particular, hydrophobic and amphiphilic molecules which are difficult or impossible to crystallize using prior art methods.

In sharp contrast to the linkers used by prior art methods, the linker configurations used by the method of the present invention:

(i) are capable of forming molecular complexes with molecules of interest of a sufficient solubility so as to facilitate crystallization thereof,

(ii) can be easily modified to include binding moieties specific for virtually any region of any molecule-of-interest,

(iii) are designed so as to direct the spatial positioning and/or orientation of bound molecules thereby facilitating crystallization thereof, and

(iv) are designed so as to provide structural rigidity to bound molecules thereby facilitating crystallization thereof.

Aside from enabling crystallization and subsequent atomic structure determination of previously uncharacterized molecules, the capacity of the present invention to multimerize and/or purify a molecule-of-interest can be advantageously applied in various biomedical fields including protein therapeutics, oral lumenal therapies for gastrointestinal diseases and self-adjuvanting or subunit vaccines.

In addition, crystallization of macromolecule pharmaceuticals, and in particular proteins, can be used to streamline manufacturing processes, as in the case with small-molecule drugs. Since a crystal is the most concentrated possible form of a protein, crystallization can be beneficial for drugs, such as antibodies, which require high doses at the delivery site. In addition, since the rate of crystal dissolution depends on its morphology, size, and the presence of excipients,

crystalline proteins may also serve as a convenient carrier-free slow release dosage form (insulin is a good example). Finally, the stability of proteins in crystalline form is higher than that of corresponding soluble or amorphous materials and, as such, crystallization can be used to greatly increase the shelf life of a drug product.

Macromolecular crystals generated according to the teachings of the present invention also find important uses as catalysts, adsorbents, biosensors and chiral chromatographic media. These may also be employed in environmental applications, including, for example, the destruction of nerve agents, for bioremediation and civil defense.

In addition to the above, the present invention provides methods of protein purification via crystal formation.

As described hereinabove, suitable GPCR-binding domains of arrestin molecules can be used to bind GPCRs with high affinity and specify. Such GPCR binding domains of arrestin molecules can therefore be used as affinity ligands for purification of such GPCRs.

Thus, according to the present invention, there is provided a method of purifying a GPCR from a sample containing a GPCR.

The method of purifying a GPCR from a sample is effected by subjecting the sample to affinity chromatography using a GPCR binding domain of an arrestin molecule.

All criteria described hereinabove regarding selection and/or modification of a GPCR binding domain of an arrestin molecule suitable as a binding domain of a molecular linker are applicable to selection and/or modification of a GPCR binding domain of an arrestin molecule suitable as a GPCR binding region of an affinity ligand for the presently described purification method. As is described in Example 10 of the Examples section below GPCR binding domains of an arrestin molecule corresponding to SEQ ID NO: 3 or SEQ ID NO: 4 can be used to efficiently bind various types of GPCRs with high specifity and affinity, and thereby to efficiently purify various GPCRs regardless of the

activation-phosphorylation state thereof.

Preferably the affinity ligand includes a purification tag for facilitating attachment of the affinity ligand to an affinity chromatography matrix.

As is described in Example 10 of the Examples section below an affinity ligand conjugated to a Strep-tag can be conveniently bound to an affinity matrix to which core streptavidin is conjugated.

Alternately, as is further described in Example 10 of the Examples section below an affinity ligand conjugated to core streptavidin can be conveniently bound to an affinity matrix to which a Strep-tag or iminobiotin is conjugated.

Suitable protocols for all phases of affinity chromatography purification of molecules are widely available in the literature of the art (see, for example: Wilchek M. & Chaiken I., 2000. Methods Mol Biol 147:1-6; Jack, G. W. Immunoaffinity chromatography. Mol Biotechnol 1, 59-86; Narayanan SR., 1994. Journal of Chromatography A 658:237-258; Nisnevitch M. & Firer MA., 2001. J Biochem Biophys Methods 49:467-80; Janson JC. & Kristiansen T. in Packings and Stationary Phases in Chromatography Techniques (ed. Unger, K. K.) 747 (Marcel Dekker, New York, 1990); Clonis, Y. D. in HPLC of Macromolecules A Practical Approach 157 (IRL Press, Oxford, 1989); Nilsson J. *et al.*, 1997. Protein Expr Purif. 11:1-16).

Preferably, the present invention is used to purify a GPCR such as rhodopsin or a class A GPCR.

Preferably, the present invention is used to purify a class A GPCR such as m2 muscarinic cholinergic receptor.

Additional objects, advantages and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771; and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To

Methods And Applications", Academic Press, San Diego, CA (1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization – A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below.

EXAMPLE 1

Generation of ordered crystals of molecules of interest by complexation thereof with non-polypeptidic molecular linkers

In order to facilitate ordered crystallization and atomic structure determination of a molecule-of-interest, non-polypeptidic molecular linkers were designed having the capacity to form a crystallizable molecular complex with molecules of a molecule-of-interest and, preferably, with a metal atom.

Materials and Methods:

Molecular linkers are generated to facilitate ordered crystallization of molecules-of-interest having the following characteristics: (a) the ability to homomultimerize molecules-of-interest in selected geometric configurations, thereby facilitating ordered crystallization of molecules-of-interest which do not naturally aggregate in configurations suitable therefor; (b) sufficient conformational rigidity so as to facilitate ordered crystallization or ordered assembly of molecules-of-interest lacking sufficient conformational rigidity therefor; (c) sufficient hydrophilicity so as to facilitate solubilization in polar solvents, and thereby crystallization, under standard crystallization-inducing conditions of molecules-of-interest lacking sufficient hydrophilicity therefor, (d)

binding moieties specific for desired regions of molecules-of-interest, thereby facilitating multimerization of the molecules-of-interest; and (c) the ability to specifically bind a metal atom being capable of facilitating 3D crystallographic analysis of molecules-of-interest by enabling generation of initial phases for X-ray diffraction crystallography. A modular organization of such molecular linkers is schematized in Figure 1a.

Such linkers may extend a binding moiety from a multimerization scaffold via a first chain of 1–3 carbon or oxygen atoms, representative examples of which are depicted in Figure 1b. These chains preferably terminate in a functional group such as [$-CO_2H$], [-OH], [$-NH_2$], [$-CO_2$], [-CONH], [-O], [-OCO] or [-NHCO] which are used to attach, via conventional ester, amide or ether formation, a second chain of suitable length and geometry so as to enable attachment of monomers of a molecule-of-interest to the multimerizing scaffold of the molecular linker in the desired spatial configuration. Such chains preferably include a molecular group, such as [$-(CH_2CH_2O)_2$-O-CH_2CH_2-] or [$-(CH_2)_4-$], to which is attached the binding moiety. Such chains possess sufficient conformational rigidity and/or hydrophilicity so as to facilitate crystallization of molecules of a molecule-of-interest complexed therewith lacking such conformational rigidity and/or hydrophilicity, respectively.

Moieties specific for binding molecules of interest are preferably polypeptides capable of directly or indirectly mediating specific recognition of a molecule-of-interest, such as core streptavidin, peptide tags or antibodies. Alternatively, molecules such as [$-N^+(CH_3)_3$] or [$-CO(CF_3)$] can be employed to specifically bind a molecule-of-interest. Binding of metal atoms to molecular linkers can be effected via the use of molecular linkers comprising multimerization scaffolds based on molecules, such as porphyrin or hydroxime, which can bind metal atoms such as Pt^{2+}, Ni^{2+}, Cu^{2+} or Co^{2+}.

Examples of molecular linkers capable of forming a crystallizable molecular complex with a molecule-of-interest and specifically binding a metal atom include, for example, porphyrin-based molecular linkers (Figures 2a and

2b, respectively) or hydroxime-based molecular linkers (Figure 3).

Thus, the molecular linkers of the present invention form molecular complexes with molecules of a molecule-of-interest being positioned in a selected spatial geometry facilitating crystallization thereof. Such molecular linkers further facilitate crystallographic analysis of a molecule-of-interest by incorporating within the crystallizable molecular complex a metal atom used to generate initial phases during X-ray crystallography.

EXAMPLE 2

Chemical synthesis of porphyrin-based molecular linkers

As described in Example 1, porphyrin-based molecular linkers can be employed to facilitate crystallization of molecules of interest by multimerizing these within substantially conformationally rigid and/or hydrophobic crystallizable molecular complexes. Such linkers further facilitate determination of the atomic structure of molecules of interest by incorporating a platinum atom which can be employed to generate initial phases during X-ray crystallographic analysis of crystals of such molecular complexes.

Synthetic procedures to generate crystallizable molecular complexes with porphyrin-based molecular linkers are depicted in Figures 4a and 4b.

The steps involved in synthetic processes to generate a porphyrin-based molecular linker, 5, 15-Di(2, 6-di(ethoxycarbonymethoxy))porphyrinato-platinum (Figure 4b, Product No. 4), and the attachment of various molecular spacers/binding domains thereto are outlined below:

Materials and Methods:

Synthesis of 5, 15-Di(2, 6-di(ethoxycarbonymethoxy))porphyrin (Product No. 3): Dipyrrolmethane (280 mg, 1.9 mmol) and 2, 6-di(ethoxycarbonymethoxy)-benzaldehyde (590 mg, 1.9 mmol) were dissolved in dichloromethane (300 ml) and purged with nitrogen. To this was added trifluoroacetic acid (75 ml, 1 mmol) and the solution was stirred for 3 hours at

room temperature. DDQ (450 mg, 2 mmol) was added, the mixture was stirred for 1 hour and neutralized with triethylamine (1.5 ml). The resultant mixture was purified by chromatography on a silica column, eluting with dichloromethane. The product was eluted as a purple band from the column and was obtained by evaporation of the eluate to give purple crystals (250 mg) of the product.

Synthesis of 5, 15-Di(2, 6-di(ethoxycarbonymethoxy))-porphyrinato-platinum (Product No. 4, where X=OCH_2CO_2Et, R=H, n=2M=Pt): The product of the previous reaction (250 mg) was dissolved in acetic acid (50 ml) and to this was added dipotassium tetrachloroplatinate (112 mg) and the mixture was refluxed for 10 min. The mixture was cooled and water (20 ml) was added. The product (350 mg) was filtered off and washed with 50 % aqueous ethanol.

Synthesis of 5, 15-Di(2, 6-di(ethoxycarbonymethoxy))porphyrinato-platinum (Product No. 4, where X=OCH_2CO_2H, R=H, n=2, M = Pt): The product of the previous step (350 mg) was suspended in 50 % aqueous ethanol (50 ml) containing sodium hydroxide (500 mg) and refluxed for 3 hrs. The mixture was then acidified drop-wise with concentrated HCl, to pH 1 and the product (280 mg) was filtered off.

Synthesis of 5, 15-Di(2, 6-di((N-biotinylaminopropyl)amidocarbonymethoxy)) porphyrinatoplatinum (Product No. 4, where X=$OCH_2CO_2NH(CH_2)_3NH$(biotinyl), R=H, n=2, M = Pt): 350 mg, 288 mmol of the product of the previous reaction was added to a solution of DCC (72 mg) in dioxane (100 ml) containing a catalytic amount of hydroxybenzotriazole (5 mg). 3-(biotinylamino)-propylamine (95 mg, 320 mmol) was then added and the mixture was stirred overnight at room temperature and filtered. The residue was washed with ethyl acetate and the filtrate was evaporated to give the crude product (605 mg). The product was then be further purified by chromatography on a silica gel column, eluting with ethyl acetate. Analogues of this compound are synthesized similarly.

The number of moieties specific for the molecule-of-interest are given by the index *n*. The steric encumbrance between such moieties determine the geometry of the molecular scaffold, and thus the geometry of the molecule-of-interest-linker complex. The biotinyl moiety described above can be used, for example to bind any molecule-of-interest which has been fused to streptavidin.

EXAMPLE 3

Chemical synthesis of a hydroxime-based molecular linker

A synthetic procedure to generate a hydroxime-based molecular linker for binding two molecules of a molecule-of-interest, thereby generating a crystallizable molecular complex containing the molecule-of-interest, is depicted in Figure 5. Such a molecular linker further facilitates determination of the crystal structure of the molecule-of-interest by chelating a copper atom which is employed to generate initial phases during X-ray crystallographic analysis of a crystal of the molecular complex.

Materials and Methods:

Synthesis of 5-((2-trimethylammonium-ethoxy)digolyloxycarbonyl)-2-hydroxyacetophenone oxime dichloride (intermediate No. 5 where $X=CO.(OCH_2CH_2)_3N^+Me_3$, $R'=Me$, $n=1$***):*** 5-Carboxy-2-hydroxyacetophenone oxime (1 g, 6 mmol) was dissolved in dioxane (50 ml) containing DCC (0.95 g) and (2-trimethylammonium-ethoxy)-digol chloride (1.4 g) dissolved in dioxane (20 ml) and the mixture was stirred for 6 hours at room temperature. The mixture was filtered and the filtrate was evaporated to dryness. The residue was then dissolved in water and the product was purified by ion exchange chromatography on a Dowex cation exchange column and was obtained as a viscous oil, on evaporation under high vacuum, as a chloride salt.

Synthesis of Bis-[5((2-trimethylammonium-ethoxy)-digolyloxy-carbonyl)-carboxy-2-hydroxyacetophenone oxime] copper (II) chelate dichloride (Product No. 6, where $X=CO.(OCH_2CH_2)_3N^+Me_3$, $R'=Me$, $n=1$, $M=Cu$***):*** 100 mg of the previous reaction product was dissolved in water (10 ml) and to this was added an aqueous solution of copper (II) chloride (1.5 ml of 0.1M solution). The solution was stirred for 4 hours and the mixture was evaporated to dryness, under high vacuum, to yield the product (110 mg) as a green solid. Analogues of this compound are synthesized similarly.

The quaternary ammonium moiety is employed to bind any molecule which is known to bind positively charged groups via cation-π interactions, such as acetylcholinesterase.

EXAMPLE 4

Synthesis of a non-polypeptidic molecular linker with biotinylated moieties for attachment of a molecule-of-interest coupled to a biotin-binding molecule

A modular system where a single type of molecular linker may bind a range of molecules of interest is highly desirable since this obviates the requirement of synthesizing a dedicated linker for each molecule-of-interest. This is effected, for polypeptides of interest, for example, by incorporating within the molecular linker and the polypeptide of interest heterologous moieties, such as polypeptides, that specifically bind to each other.

Since one of the highest binding affinities known between any two non-covalently associated molecules is that between core streptavidin and biotin, the use of such binding a pair is ideal for binding a molecule-of-interest to a molecular linker. Such a binding interaction serves to optimize crystallization of the molecule-of-interest since it facilitates formation of a highly stable and rigid molecular complex which can be easily crystallized.

The synthetic process for linkage of a biotin moiety to a porphyrin-based molecular linker is outlined in Figure 6a and is performed as follows:

Synthesis of 5, 10, 15, 20-tetra-(3-ethoxycarbonyl)porphyrin (Product No. 2), where X = OCH_2CO_2Et, n = 1): Ethyl 3-formylbenzoate (5 g) and pyrrole (2 g) were dissolved in chloroform (1 liter) and the solution was purged with nitrogen for 10 min. A solution of $BF_3.Et_2O$ (4 ml of 2.5M solution). After 1 hour chloranil (5.4 g) was added and the mixture was refluxed for 1 hour. The mixture was cooled to room temperature and 1 equivalent of triethylamine was added. The solution was evaporated to dryness to give the crude product, which was washed with methanol three times. The product remained as a purple solid (1.43 g). The product was then elaborated, analogously to the method described above for synthesis of porphyrin-based molecular linkers, into further examples of the invention.

EXAMPLE 5

Synthesis of a hydroxime-based molecular linker with trimethylammonium moieties for attachment of molecules of a molecule-of-interest

In order to bind molecules of a molecule-of-interest in the desired spatial configuration within a crystallizable molecular complex a molecular linker, according to the method of the present invention, must be of a suitable dimension and geometry.

Such positioning of a molecule-of-interest within a crystallizable molecular complex is effected, for example, by employing molecular linkers with a hydroxime-based multimerization scaffold, as described above, to which molecules of a molecule-of-interest are attached via trimethylammonium moieties. As well as allowing binding of molecules of interest to a molecular linker without steric hindrance, trimethylammonium, being of substantial hydrophilicity and conformational rigidity, further facilitates solubilization and crystallization, respectively, of the molecular complex.

The chemical attachment of trimethylammonium to a hydroxime-based molecular linker is depicted in Figure 6b. As described above, inclusion of a metal atom within the hydroxime-based molecular linker facilitates determination

of the atomic structure of the molecule-of-interest by providing initial phases during X-ray crystallographic analysis of a crystal of a molecular complex including a molecule-of-interest.

EXAMPLE 6

Crystallizable molecular complexes comprising a mutagenesis polypeptide of interest and a heterologous molecular linker

Mutagenesis of a polypeptide of interest is employed so as to optimize the crystallizability of a molecular complex formed by a linker therewith.

The polypeptide of interest is mutagenized in order to adjust the steric fit between the molecular linker and the molecules of the polypeptide of interest. Such an adjustment is employed in order to optimize the number and/or physico-chemical characteristics of the crystal contacts of the crystallizable molecular complex formed by association of molecules of the polypeptide of interest with the molecular linker. Additionally, selected residues of the polypeptide of interest are mutagenized in order to optimize the solubility and/or rigidity of the crystallizable molecular complex formed by association of molecules of the polypeptide of interest with the molecular linker.

Acetylcholinesterase (AChE) and muscarinic acetylcholine receptor (mAChR) are molecules which are well characterized pharmacologically and AChE is known to crystallize in a series of well-characterized lattices. Thus, AChE is mutagenized so as to optimize its packing within a molecular linker when multimerized therewith.

Muscarinic acetylcholine receptor, whose 3D structure remains to be determined, is representative of a broad class of integral membrane proteins of great pharmacological importance. However, it is known to bind ligands possessing a similar structure to those binding AChE. Thus a modified molecular linker, based on the one employed for crystallization of mutagenized AChE, as described above, is employed in order to crystallize mAChR, an integral membrane protein.

Materials and Methods:

The molecule-of-interest is mutagenized via standard recombinant techniques and is produced using a bacterial expression system. The purified protein is solubilized in a monodisperse solution according to standard crystallization procedures available in the literature. To this solution, a suitable amount of molecular linker is added. A 5 microliter aliquot of this molecular linker solution is added to 5 microliters of mother solution on a siliconized glass coverslip (18-22 mm diameter). The coverslip is placed over a well containing a solution buffered at the appropriate pH and adjusted to the optimal concentration of precipitants (e.g. PEG 5000 or ammonium sulfate). The drop is allowed to equilibrate at the appropriate temperature (e.g. 20° C) for an amount of time necessary for the crystal to form.

EXAMPLE 7

Crystallization of a molecule-of-interest by complexation with a molecular linker composed of a homomultimerizing molecule conjugated to a modular recognition domain specific for a molecule-of-interest

One of the most versatile, convenient and specific means of specifically binding a molecule-of-interest is via antibodies.

Therefore, molecular linkers were designed consisting of a chimeric polypeptide composed of fused scFv, core streptavidin and histidine tag segments, as depicted schematically in Figure 7a. Such single-chain Fv-core streptavidin chimeric polypeptides and polypeptides including histidine tags have been previously described (Ladner, R.C. *et al.*, US patent 4,946,778) and (Sheibani N., 1999. Prep Biochem Biotechnol. 29(1):77), respectively. The relative positions of the single-chain Fv molecule and the core streptavidin segments can also be inverted. The peptide sequences GSAA (SEQ ID NO: 1) and GS (SEQ ID NO: 2) are inserted between the V_L and core streptavidin, and between the core streptavidin and the His-tag domains, respectively, so as to provide the required flexibility for appropriate folding of the fusion protein.

Optionally, association of a metal atom with the crystallizable molecular complex is effected via the use of a second chimeric polypeptide comprising Strep-tag, metal atom-binding and purification tag segments, as depicted in Figure 7b. The Strep-tag domain of this chimera serves to bind the core streptavidin domain of the core streptavidin-containing chimera described hereinabove and thus serves to associate the molecule-of-interest with a metal atom binding molecule. Binding of the metal atom to the metal atom binding domain is effected either prior to, concomitantly or following the binding steps described above. Furthermore, the purification tag of the metal atom binding chimera can be employed to perform the same functions as the purification tag comprised in the core streptavidin-containing chimera described above. The conformation of a tetramerized complex obtained using the above-described system is depicted in Figure 8.

Such a molecular linker thus binds a molecule-of-interest via its scFv domain, tetramerizes via its core streptavidin domain and can be easily identified by immunoblotting analysis or purified by affinity chromatography, either prior to or following binding of a molecule-of-interest, via its purification tag domain.

One advantage of utilizing streptavidin as the core of molecular linkers, is that extensive literature exists for the expression and purification of streptavidin itself (Wu SC. *et al.*, 2002. Protein Expression and Purification 24:348-356; Gallizia A. *et al.*, 1998. Protein Expression and Purification 14:192-196) and of streptavidin fusion proteins (Sano T. & Cantor CR. 2000. Methods Enzymol. 326:305-11). Smaller and more stable streptavidins than the native form have been produced recombinantly (Sano T. *et al.*, 1993. Journal of Biological Chemistry 270:28204-28209) and the gene sequence has been optimized for expression in *E. coli* (Thompson LD. & Weber PC., 1993. Gene 136:243-6). The tetramer of these smaller "cores" displays enhanced stability under denaturing conditions, and their biotin binding sites appear to be more accessible. A small core size is also preferable, as it helps to keep the size of the final polypeptidic molecular linker to a minimum, making the scaffold easier and cheaper to

produce and purify. Smaller molecular linkers may be advantageous since, as a rule of thumb, a smaller and tightly packed multimerization scaffold will introduce less disorder in the final crystallization complex, thus ensuring optimal ordering of crystals.

Crystallization of a molecule-of-interest using the above-described molecular linkers is achieved as follows:

The chimeric polypeptide described above is produced in a first step via standard recombinant DNA, protein expression and protein purification techniques. In a second step, the molecule-of-interest is crystallized within a crystallizable molecular complex formed by tetramerization of the chimera via core streptavidin, thereby generating a molecular linker, and by binding of molecules of the molecule-of-interest to the scFv domains of the molecular linker.

The order in which these various non-covalent binding steps are effected can be essentially shuffled at will since these involve biological interactions occurring under similar physiological conditions. As discussed above, incorporation of a metal atom into a molecular complex containing a molecule-of-interest serves to facilitate solution of the 3D atomic structure of the molecule-of-interest.

The scheme outlined hereinabove for crystallization of a molecule-of-interest is highly modular and flexible and the components thereof are interchangeable while retaining the basic functionalities required for formation of a crystallizable molecular complex. For example, the molecule-of-interest-specific scFv domain is exchangeable with any other molecule specifically binding the molecule-of-interest. One such example is a toxin specific for a membrane receptor, as described in the embodiments of the present invention. This is effected by employing the genetic sequence encoding the toxin instead of that of the scFv during the recombinant DNA manipulation phase of this crystallization method. Similarly, the metal atom binding segment of the chimera described above is exchangeable, via chemical synthesis, with a

non-polypeptidic metal chelating molecule, such as porphyrin or hydroxime described in Examples 4 and 5, respectively. When employing appropriate combinations of auxiliary functional domains within the molecular linker, the core streptavidin domain segment of the molecular linker is exchangeable with any other suitable homomultimerizing molecule.

An alternative method for association of a metal atom with the crystallizable molecular complexes of the present invention involves the use of a molecular linker composed of a single type of molecule which includes the metal atom binding segment as well as the molecule-of-interest-binding, homomultimerizing and purification tag segments. This is effected, for example, via a chimeric polypeptide including all these functional segments.

Thus, such molecular linkers can be employed to facilitate crystallization and 3D atomic structure determination of a molecule which can be bound by an antibody.

EXAMPLE 8

Generation of ordered crystals of a polypeptidic molecule-of-interest via expression as a fusion chimera with a heterologous homomultimerization domain

In order to crystallize a polypeptidic molecule-of interest, the molecule-of-interest is expressed as a fusion chimera with a purification tag, such as an epitope tag, which is specifically bound by a purification tag-binding molecule utilized as the molecule-of-interest binding moiety of the molecular linker.

Such a crystallization system presents the advantage of enabling a single molecular linker to facilitate the crystallization of any polypeptide-of-interest, modified as described above.

All alternatives described in Example 7 above pertaining to functional segments of molecular linkers, and to methods of including metal atoms in crystallizable complexes are applicable to the presently disclosed method.

Production of a chimeric polypeptide comprising the molecule-of-interest and the tag is effected by cloning nucleic acid sequences encoding the molecule-of-interest into a bacterial expression vector which comprises a nucleic acid sequence encoding the tag, and which is configured to express the molecule-of-interest and the tag in-frame as a fusion protein.

Suitable bacterial strains are transformed with the expression vector, and recombinant chimera produced by transformants is recovered using standard recombinant protein technology, and is crystallized using standard crystallization conditions for X-ray crystallography.

Thus, this method provides a means of facilitating the crystallization and crystallographic analysis of a broad range of polypeptides of interest conjugated to a heterologous molecule via a single type of molecular linker.

EXAMPLE 9

Generation of crystals of G protein coupled receptors suitable for determination of three dimensional atomic structure thereof

A very large number of human diseases are associated with G protein coupled receptor disfunction, as illustrated by the fact that G protein-coupled receptors constitute the most prominent family of drug targets, as described above. Nevertheless, pharmacological treatment of diseases associated with GPCRs remains suboptimal, however. Thus, there is a very great need for novel GPCR specific drugs. One way to generate such drugs would be to elucidate the 3D atomic structure of GPCRs at high resolution so as to enable the rational design of pharmacological agents capable of having a desired regulatory effect on the activity of such receptors. However, prior art methods cannot be used to efficiently generate crystals of membrane proteins such as GPCRs, which crystals being suitable for determining the 3D atomic structure of such receptors at high resolution. In order to fulfill this important need, the present inventors have designed molecular linkers capable of being used to generate highly ordered, X-ray crystallography grade crystals of G protein coupled receptors

suitable for X-ray crystallographic analysis of the 3D atomic structure of such receptors as follows.

Background:

Streptavidin: Streptavidin is a 159 amino acid residue protein produced by *Streptomyces avidinii* that binds up to four molecules of biotin with ultra-high affinity ($K_d \sim 10^{-15}$ M; Green NM., 1990. Methods in Enzymology 184:51-67), to form an ultra-stable homotetramer that does not dissociate even in the presence of 6 M urea (Kurzban GP., 1991. J Biol Chem. 266, 14470-14477). The crystallographic structure of core streptavidin illustrates that each streptavidin monomer folds into an eight-stranded antiparallel β-barrel, with the biotin binding site built by residues of the barrel itself and a loop of an adjacent subunit to form a very stable dimer (Freitag S. *et al.*, 1997. Protein Science 6:1157-1166). Extensive intersubunit contacts between the dimers give rise to the final tetrameric structure having tight quaternary assembly and fixed geometry (Green NM., 1990. Methods in Enzymology 184:51-67).

Another advantage of using streptavidin as the core of a molecular linker, is that extensive literature exists for the expression and purification of streptavidin itself (Wu SC. *et al.*, 2002. Protein Expression and Purification 24:348-356; Gallizia A. *et al.*, 1998. Protein Expression and Purification 14:192-196), and of streptavidin fusion proteins (Sano T. & Cantor CR. 2000. Methods Enzymol. 326:305-11). Smaller and more stable streptavidins than the native form have been produced recombinantly (Sano T. *et al.*, 1993. Journal of Biological Chemistry 270:28204-28209) and the gene sequence has been optimized for expression in *E. coli* (Thompson LD. & Weber PC., 1993. Gene 136:243-6). The tetramer of these smaller cores displays enhanced stability under denaturing conditions, and their biotin binding sites appear to be more accessible. A small core size is also preferable, as it helps to keep the size of the final polypeptidic molecular linker to a minimum, making the scaffold easier and cheaper to produce and purify. Smaller molecular linkers may be advantageous since, as a rule of thumb, a smaller and tightly packed multimerization scaffolds

will introduce less disorder in the final GPCR-linker complex, thus ensuring higher quality crystals.

Arrestins: The arrestin family consists of visual arrestin (v-arrestin, S-arrestin), cone-arrestin, β-arrestin (β-arrestin-1 and arrestin-2), and β-arrestin-2 (arrestin-3). V- and cone-arrestins are exclusively expressed in rod and cone photoreceptors, respectively, and are highly specialized to bind specifically to rhodopsin, or cone cell pigments. The two closely related β-arrestins are ubiquitously expressed and are responsible for the termination of the primary signaling event for most, if not all, class I (rhodopsin-like) GPCRs.

At the sequence level, visual arrestin is 60 % identical to the β-arrestins, which show 78 % sequence identity between themselves. The three dimensional structure of v-arrestin (Hirsch JA. *et al.*, 1999. Cell 97:257-69; Granzin, J. *et al.*, 1998. Nature 391:918-21) and of β-arrestin (Han M. *et al.*, 2001. Structure (Camb) 9:869-80) have been solved and reported in the literature.

Arrestins bind with subnanomolar affinities (Gurevich VV. *et al.*, 1995. Journal of Biological Chemistry 270:720-731) exclusively to agonist-activated GPCRs that have been phosphorylated by G protein-coupled receptor kinases (GRKs) on serine and threonine residues located in the third intracellular loop or carboxyl terminal tail (Gurevich VV. & Benovic JL., 1992. Journal of Biological Chemistry 267:21919-21923; Lohse M. *et al.*, 1992. J Biol Chem. 267:8558-8564; Lohse MJ. *et al.*, 1990. Science 248:1547-50). The association of a single arrestin with a GRK-phosphorylated receptor uncouples the receptor from its cognate G protein, resulting in the termination of GPCR signaling, a process termed desensitization (Gurevich VV. & Benovic JL., 1992. Journal of Biological Chemistry 267:21919-21923; Lohse M. *et al.*, 1992. J Biol Chem. 267:8558-8564; Lohse MJ. *et al.*, 1990. Science 248:1547-50; Pippig S. *et al.*, 1993. Journal of Biological Chemistry 268:3201-3208; Attramadal H. *et al.*, 1992. J Biol Chem. 267:17882-17890). In the case of β-arrestins, these molecules then target desensitized receptors to clathrin-coated pits for endocytosis by functioning as adaptor proteins that link the receptor to

components of the endocytic machinery such as AP-2 and clathrin (Goodman, OB Jr. *et al.*, 1996. Nature 383:447-50; Laporte SA. *et al.*, 1999. Proc Natl Acad Sci U S A. 96:3712-3717; Laporte SA. *et al.*, 2000. J Biol Chem. 275:23120-23126; Ferguson SSG. *et al.*, 1996. Science 271:363-366). The internalized receptors are dephosphorylated in endosomes and recycled back to the cell surface fully resensitized (Zhang L. *et al.*, 1997. J Biol Chem. 272:14762-8; Oakley RH. *et al.*, 1999. J Biol Chem. 274:32248-57; Krueger KM. *et al.*, 1997. J Biol Chem 272:5-8).

The overall structures of β-arrestins and v-arrestin share many similar features: all are elongated molecules with a central polar core built by a network of charge-charge interactions (amino acid residues 1–8, 30, 175–176, 296, 303 and 382; where the numbering follows the sequence of v-arrestin) flanked by the N (amino acid residues 8–180) domain, C domain (amino acid residues 188–362) and a C tail (amino acid residues 372–404) that tightly interacts with the two domains and with the N terminus. Residues 98–108 in the N-domain form a cationic amphipathic α-helix that might serve as a reversible membrane anchor. Structural variations between arrestins are mostly found in surface loops. Analysis of β-arrestin and v-arrestin structures has shown that such arrestins are characterized by a very similar overall structure (Han M. *et al.*, 2001. Structure (Camb) 9:869-80). The loop regions that vary between β-arrestin and v-arrestin also vary between different crystal forms of the same protein, reflecting the intrinsic flexibility of those regions rather than inherent structural differences between the two arrestins, as can be seen from the distribution of B factors. The crystal structures of v-arrestin and of β-arrestin analyzed represent their respective inactive basal states, where the polar core is intact.

It has been shown that the predominant region of receptor binding in v-arrestin is contained within amino acid residues 90–140. A portion of this region (amino acid residues 95–140) expressed as a fusion protein with glutathione S-transferase has been shown to be capable of binding to rhodopsin regardless of the activation or phosphorylation state of the receptor (Smith WC.

et al., 1999. Biochemistry 38:2752-61). Mutations disrupting the polar core such as the v-arrestin mutant R175E, promote phosphorylation-independent binding of arrestin to the receptor (Gurevich VV. & Benovic JL. Molecular Pharmacology 51:161-169; GrayKeller MP. *et al.*, 1997. Biochemistry 36:7058-7063). Segment-swapping experiments between visual and non-visual arrestins have demonstrated that substituting amino acid residues 50-90 of v-arrestin with the equivalent element of β-arrestin (amino acid residues 46-86) can switch the binding specificity of v-arrestin to high affinity binding of activation-phosphorylated m2 muscarinic cholinergic receptor (P-m2 mAchR*) while losing the affinity for activation-phosphorylated rhodopsin (P-Rh*; Han M. *et al.*, 2001. Structure (Camb) 9:869-80). Remarkably, the single amino acid mutation V90S was shown to eliminate this difference, permitting v-arrestin to bind P-m2 mAchR* with similar affinity as β-arrestin without significant concurrent loss of its affinity to P-Rh*. In addition, elimination of the hydrophobic side chains of residues 11-13 was observed to disrupt the interaction between the N-domain and the amphipathic α-helix, and enhances phosphorylation-independent binding of arrestin (Vishnivetskiy SA. *et al.*, 2000. J Biol Chem. 275:41049-41057).

These truncation and deletion studies point to the N-terminal domain as the primary domain of interaction–the truncated N-domain of arrestin binds to P-m2 mAchR with a K_d = 2 nM (Gurevich VV. *et al.*, 1995. Journal of Biological Chemistry 270:720-731). Additional data also point to the C-domain as playing a significant role in receptor binding since a truncated form of arrestin in which just a short C-terminal region is removed displays a K_d = 1 nM (Gurevich VV. *et al.*, 1995. Journal of Biological Chemistry 270:720-731).

The evidence accumulated so far suggest two possible mechanisms promoting receptor-arrestin interaction that are independent of the specific GPCR subtype. One mechanism is linked to the polar core, where critical salt bridges keep arrestin in its basal state (Hirsch JA. *et al.*, 1999. Cell 97:257-69). An activation-phosphorylated GPCR would interact with arrestin, thereby disrupting

the polar core and triggering the conformational changes required for high-affinity receptor binding. A second general mechanism can be derived from structural and mutagenesis data, whereby receptor binding is triggered and/or enhanced by the membrane translocation of arrestin's amphipathic α-helix I (Han M. *et al.*, 2001. Structure (Camb) 9:869-80).

Materials and Methods:

The above-described data relating to streptavidin indicates that core streptavidin can be used to generate molecular linkers having a highly stable and rigid predetermined quaternary structure and geometry suitable for optimally facilitating crystallization of crystallization complexes. The above-described data relating to arrestins indicates that a polypeptide composed of amino acid residues 11–190 of human beta-arrestin-1a with mutation R169E (SEQ ID NO: 3; Figure 9a), or a polypeptide composed of amino acid residues 11–370 of human beta-arrestin-1a with mutation R169E (SEQ ID NO: 4; Figure 9b) can serve as ligands capable of binding different classes of GPCRs with high affinity and specificity regardless of the phosphorylation/activation state thereof. Mutation R169E in human beta-arrestin-1a is homologous to the above-described R175E mutation in v-arrestin, as shown by published amino acid sequence comparisons (Han M. *et al.*, 2001. Structure (Camb) 9:869-80; Hirsch JA. *et al.*, 1999. Cell 97:257-69). Mutation R169E thus enables binding of GPCRs independently of the activation-phosphorylation state thereof. There is a serine residue located at position 86 in wild-type human beta-arrestin-1a which corresponds to mutation V90S in v-arrestin as shown by the aforementioned published amino acid sequence comparisons. As described hereinabove, the presence of a serine residue at this position confers the capacity to bind multiple types of GPCRs. Thus, the polypeptides corresponding to SEQ ID NOs: 3 and 4 have the capacity to bind multiple types of GPCRs as well as the capacity to bind GPCRs independently of the activation-phosphorylation state thereof.

Thus, molecular linkers were designed incorporating a streptavidin based core and arrestin based GPCR binding portions.

Streptavidin-arrestin chimera based molecular linkers: Two polypeptidic molecular linkers for generation of X-ray crystallography grade crystals of molecular linker-GPCR complexes were designed. The first linker (SEQ ID NO: 5; Figure 10a) is composed of a chimeric protein consisting of the N- to C-terminal segments; T7 tag, core streptavidin, the peptide linker GSAA (SEQ ID NO: 1), and the above-described human beta-arrestin-1a derived polypeptide set forth in SEQ ID NO: 3. The second linker (SEQ ID NO: 6; Figure 10b) is composed of a chimeric protein consisting of the N- to C-terminal segments; T7 tag, core streptavidin, the peptide linker GSAA (SEQ ID NO: 1), and the above-described human beta-arrestin-1a segment set forth in SEQ ID NO: 4.

These molecular linkers can be conjugated to a metal atom via biotinylated porphyrin synthesized, as described above. Molecular linkers having streptavidin cores can adopt a highly stable and rigid predetermined quaternary structure and geometry suitable for optimally facilitating crystallization of crystallization complexes, and bind with high specificity and affinity the largest possible set of different GPCRs.

Streptavidin-metallothionein chimera/arrestin–Strep-tag chimera based molecular linkers: Polypeptidic molecular linkers for generation of X-ray crystallography grade crystals of molecular linker-GPCR complexes were designed using a system of two polypeptide chimeras. One chimera consists of the N- to C-terminal segments; T7 tag, core streptavidin, and metallothionein. The other chimera consists of, the N- to C-terminal segments; the above-described human beta-arrestin-1a derived polypeptide set forth in SEQ ID NO: 3 or SEQ ID NO: 4 and a Strep-tag. In this system, the arrestin comprising chimera is attached to the core of the molecular linker by specific binding of the Strep-tag, to which the arrestin derived polypeptide is fused, to the core streptavidin contained in the molecular linker. The metallothionein segment can be used to incorporate several heavy metal atoms such as Cd^{2+} in the crystallization complex for providing initial phases for analysis of X-ray crystal

diffraction data.

Metallothionein-streptavidin fusion proteins are produced essentially as previously described in the literature, with minor modifications for including the T7 tag and for adjusting the length of the streptavidin core (Sano T. *et al.*, 1999. Proc Natl Acad Sci U S A. 89:1534-8).

The T7 tag was used in order to increase production of recombinant proteins and to facilitate their purification.

The availability of the 3D structures of all proteins employed in the construction of the above-described polypeptidic molecular linkers has enabled modeling of the structure of such molecular linkers with a significant degree of confidence.

Chimeric proteins are cloned in standard expression vectors for expression of recombinant proteins in *E. coli* using standard recombinant DNA procedures on the basis of genomic DNA sequences, cDNA sequences or protein sequences of arrestins and streptavidins available in public and private databases (e.g., GenBank, EMBL, PIR, NCBI Pubmed, etc). Sequences coding for the fusion protein are codon-optimized for expression in *E. coli* (Thompson LD. & Weber PC., 1993. Gene 136:243-6). Streptavidin fusion proteins are optimally designed and produced with the streptavidin core at the N-terminus and are produced as inclusion bodies to maximize free biotin binding sites and refolding as previously described (Sano T. & Cantor CR. 2000. Methods Enzymol. 326:305-11). Introduction of the T7 tag at the N-terminus of the chimeric proteins increases expression thereof and permits easier purification thereof (Gallizia A. *et al.*, 1998. Protein Expression and Purification 14:192-196. Recombinant chimeras are purified from bacterial inclusion bodies using standard techniques and T7 tag specific affinity chromatography. The purified molecular linkers are then individually mixed with different types of GPCRs at stoichiometric ratios, and under physiological conditions suitable for enabling complex formation therebetween. Formed complexes are subsequently subjected to crystallization inducing conditions.

For a one-step purification/molecular linker complexation procedure, fusion proteins containing core streptavidin, or molecular complexes containing such fusion proteins, are bound to affinity chromatography columns with matrices conjugated to streptavidin specific ligands, and are directly eluted from such columns using biotinylated molecular linker, such as biotinylated porphyrin (described above).

The monodispersity and second virial coefficient of solutions containing molecular linkers, GPCRs, and complexes comprising molecular linkers and/or GPCRs are monitored via light scattering techniques so as to select optimal preparations thereof for crystallization (Curtis RA. *et al.*, 2001. Journal of Physical Chemistry B 105:2445-2452; Ruppert S. *et al.*, 2001. Biotechnology Progress 17:182-187; Hitscherich C. *et al.*, 2000. Protein Science 9:1559-1566).

Results:

With each of the above-described types of molecular linkers, different types of GPCRs are efficiently crystallized conjugated to heavy metal atoms suitable for generating initial phases for X-ray crystallographic analysis of 3D atomic structure. Such crystals are highly ordered, X-ray crystallography grade, crystals.

Conclusion: The above-described GPCR crystallization method can be used to generate highly purified, highly ordered, X-ray crystallography grade crystals of numerous classes of GPCRs, regardless of the activation/phosphorylation state thereof, suitable for determining the 3D atomic structure of such GPCRs. The present method is superior to all prior art methods, since prior art methods cannot be used to efficiently generate highly ordered crystals of different types of GPCRs.

EXAMPLE 10

Efficient purification of different classes of correctly folded G protein-coupled receptors via arrestin based affinity chromatography

As described in the previous Example, there is a vital need for novel

GPCR targeting drugs. In order to provide the data required for producing such drugs, pharmacological, biochemical, and structural studies must be performed on GPCRs. Such studies require significant quantities of highly purified, correctly folded GPCRs. There is therefore a need for methods of producing large quantities of various types of correctly folded GPCRs. Various prior art approaches have been attempted for purifying GPCRs. One approach, has attempted isolating and purifying GPCRs from primary tissues. Another approach has attempted to isolate and purify GPCRs via expression of such molecules as recombinant proteins in heterologous systems. However, all prior art approaches are unsatisfactory for producing satisfactory yields of correctly folded GPCRs due to the low natural abundance of GPCRs in primary tissues, and due to the lack of a suitable method of purifying GPCRs, membrane proteins whose correct folding is highly dependent on the membranal environment, in the correctly folded state. Furthermore, prior art approaches cannot be used to efficiently purify multiple GPCR types. For example, purification tag based purification systems cannot discriminate between folded and unfolded states of tagged proteins, and furthermore are restricted by the requirement that the tag be accessible on the surface of the protein, and not buried within the protein. Affinity purification techniques based on monoclonal antibodies or specific receptor ligands require cumbersome testing and preparation, are expensive, and are typically dedicated to a single type of target molecule. Thus, all prior art approaches have failed to provide an adequate solution for efficient production of purified, correctly folded, GPCRs of various types. In order to fulfill this important need, the present inventors have devised a novel and improved method of isolating GPCRs as follows.

Materials and Methods:

The capacity of the above-described arrestin-derived polypeptides (SEQ ID NOs: 3 and 4) to bind numerous classes of GPCRs regardless of the activation-phosphorylation state thereof indicates that such polypeptides constitute ideal capture ligands for affinity chromatography of a wide range of

GPCRs. Such forms of arrestin are used for affinity chromatography purification of GPCRs as follows.

Each of the above-described GPCR-binding human beta-arrestin-1a derived polypeptides (SEQ ID NOs: 3 and 4) is synthesized via standard recombinant protein production techniques, and is individually coupled to a suitable affinity purification support matrix such as an agarose, polyacrylamide, silica, cellulose or dextran matrix (Wilchek M. & Chaiken I., 2000. Methods Mol Biol 147:1-6; Jack, GW., 1994. Mol Biotechnol. 1:59-86; Narayanan SR., 1994. Journal of Chromatography A 658:237-258; Nisnevitch M. & Firer MA., 2001. J Biochem Biophys Methods 49:467-80; Janson JC. & Kristiansen T. in Packings and Stationary Phases in Chromatography Techniques (ed. Unger, K. K.) 747 (Marcel Dekker, New York, 1990)).

The GPCR-binding polypeptides are coupled to the support matrix covalently and in an orientation specific manner via a standard coupling reaction (see, for example: Wilchek M. & Chaiken I., 2000. Methods Mol Biol 147:1-6; Jack GW., 1994. Mol Biotechnol. 1:59-86; Narayanan SR., 1994. Journal of Chromatography A 658:237-258; Nisnevitch M. & Firer MA., 2001. J Biochem Biophys Methods 49:467-80; Clonis YD. in HPLC of Macromolecules A Practical Approach 157 (IRL Press, Oxford, 1989)).

Alternatively, GPCR-binding polypeptides are produced fused to a Strep-tag (Schmidt TGM. *et al.*, 1996. Journal of Molecular Biology 255:753-766; Skerra A. & Schmidt TGM., 1999. Biomolecular Engineering 16:79-86), as previously described (Nilsson J. *et al.*, 1997. Protein Expr Purif. 11:1-16), and is coupled to a support matrix conjugated to streptavidin.

As a further alternative, the arrestin segment is produced fused to an N-terminal core streptavidin moiety and is a coupled to a support matrix conjugated with Strep-tag peptide or iminobiotin (Sano T. *et al.*, 1998. Journal of Chromatography B 715:85-91).

An affinity chromatography column is prepared using the arrestin-conjugated matrix, a sample containing a soluble GPCR is applied to the

column, the column is subjected to a cycle of washes for removal of contaminants, and fractions are eluted using a suitable buffer. Free GPCR is then eluted using a buffer containing a peptide that specifically competes with GPCR for binding with arrestin (Gurevich VV. *et al.*, 1995. Journal of Biological Chemistry 270:720-731; Smith, W. C. *et al.*, 1999. Biochemistry 38:2752; Raman D. *et al.*, 1999. Biochemistry 38:5117-23; Bennett TA. *et al.*, 2001. J Biol Chem. 276:22453-60; Sternemarr R. *et al.*, 1993. Journal of Biological Chemistry 268:15640-15648); tagged arrestin-GPCR complex is eluted using a standard buffer specific for uncoupling the tag from its matrix-conjugated ligand (Nilsson J. *et al.*, 1997. Protein Expr Purif. 11:1-16); or streptavidin-arrestin fusion protein is eluted with biotin, or a biotinylated molecule, such as biotinylated porphyrin, as described in the preceding Example, thereby enabling simultaneous purification and molecular linker complexation thereof. Elution of GPCR as a complex with the arrestin ligand is advantageous for obtaining correctly folded GPCR in high yield due to arrestin functioning as a stabilizing adjuvant to the receptor preparation (Hulme EC. & Curtis CA., 1998. Biochemical Society Transactions 26:S361) Separation of GPCR from tagged arrestin is then effected using the aforementioned peptide that specifically competes with the GPCR for binding with arrestin.

Purification of GPCR in eluted fractions is monitored via standard light scattering techniques.

The above described procedure is repeated using different classes of unmodified or suitably modified GPCRs using the same type of, or the same suitably recycled, purification column.

Results:

Significant quantities of highly purified, correctly folded GPCRs of numerous classes are produced.

Conclusion: The above-described method of the present invention can be used conveniently and rapidly produce large quantities of highly purified, correctly folded GPCRs of different classes. Such purified GPCRs can be used

to obtain valuable information required for generating novel GPCR-targeting drugs. As such, the method of the present invention is significantly superior to prior art methods which cannot be used to efficiently purify various types of correctly folded GPCRs in significant quantities.

EXAMPLE 11

Universal molecular linkers for crystallization of histidine-tagged membrane proteins

Solution of the 3D structure of membrane proteins, is crucial for the rational design of drugs targeting such proteins. To date, X-ray diffraction analysis of highly ordered crystals comprising such proteins remains the only way to solve the 3D atomic structure of such proteins. However, no prior art crystallization methods can be used to efficiently generate such crystals. In order to fulfill the critical need for such methods, the present inventors have devised universal molecular linkers for crystallizing essentially any histidine tagged membrane protein.

Materials and Methods:

Crystallization via porphyrin-NTA-Ni^{2+} molecular linker: A porphyrin based molecular linker comprising N-(5-amino-1-carboxypentyl)imino-diacetic acid (NTA) groups is synthesized and is chelated to Ni^{2+} using standard chemical techniques. A schematic diagram of porphyrin-NTA-Ni^{2+} molecular linker is shown in Figure 11. A sample containing a recombinant histidine tagged membrane protein displaying an accessible histidine tag is generated using standard techniques (e.g., refer to Sheibani N., 1999. Prep Biochem Biotechnol. 29:77). The sample containing the histidine-tagged membrane protein is reacted with porphyrin-NTA-Ni^{2+} in the appropriate stoichiometry and under suitable reaction conditions for formation of complexes of porphyrin-NTA-Ni^{2+} and the histidine-tagged protein. Complexation occurs via association of the chelated nickel ion with the histidine tag of the membrane protein. The complex is purified, dissolved in a suitable buffer, and is crystallized using standard

crystallization conditions.

The above described process is repeated using different histidine-tagged membrane proteins.

Crystallization via anti histidine tag single-chain Fv-core streptavidin fusion protein molecular linker: In order to crystallize a membrane protein-of-interest, a polypeptidic molecular linker composed of a fusion protein comprising, from N- to C-terminal; anti histidine tag single chain Fv derived from monoclonal antibody 3D5 (Kaufmann, M. *et al.*, 2002. J Mol Biol. 318. 135-47) and core streptavidin is generated. The recombinant single chain Fv-core streptavidin chimera is produced as previously described, with minor modifications (see, for example: Cloutier SM. *et al.*, 2000. Molecular Immunology 37:1067-1077; Dubel S. *et al.*, 1995. J Immunol Methods 178:201; Huston JS. *et al.*, 1991. Methods in Enzymology 203:46; Kipriyanov SM. *et al.*, 1995. Hum Antibodies Hybridomas 6:93; Kipriyanov SM. *et al.*, 1996. Protein Engineering 9:203; Pearce LA. *et al.*, 1997. Biochem Molec Biol Intl 42:1179-1188). The membrane protein-of-interest is produced as a recombinant histidine tagged protein displaying an accessible histidine tag using standard techniques (e.g., refer to Sheibani N. 1999. Prep Biochem Biotechnol. 29:77). A sample containing the histidine-tagged membrane protein-of-interest is reacted with the single chain Fv-core streptavidin molecular linker in an appropriate stoichiometry under suitable reaction conditions for formation of complexes of the molecular linker and the histidine-tagged protein (refer, for example to: Kaufmann, M. *et al.*, 2002. J Mol Biol. 318. 135-47). The complex is purified, dissolved in a suitable buffer, and is crystallized using standard crystallization conditions.

Results:

Highly ordered, X-ray crystallography grade crystals, each containing a different membrane protein, are efficiently generated using both porphyrin-NTA and anti histidine tag single-chain Fv-core streptavidin based molecular linkers.

Conclusions: The above-described molecular linkers can be used to

efficiently generate different highly ordered, X-ray crystallography grade crystals, each comprising a different membrane protein. Such crystals can be used to determine the 3D atomic structure of such membrane proteins. As such the method of the present invention is superior to all prior art methods of generating membrane proteins since these cannot be used to efficiently generate highly ordered crystals of membrane proteins.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents, patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent, patent application or sequence identified by their accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A method of generating a crystal containing a molecule-of-interest, the method comprising:

(a) contacting molecules of the molecule-of-interest with at least one type of heterologous molecular linker being capable of interlinking at least two molecules of the molecule-of-interest to thereby form a crystallizable molecular complex of defined geometry; and

(b) subjecting said crystallizable molecular complex to crystallization-inducing conditions, thereby generating the crystal containing the molecule-of-interest.

2. The method of claim 1, wherein said at least one type of heterologous molecular linker is selected such that said crystallizable molecular complex formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

3. The method of claim 1, wherein the molecule-of-interest is a polypeptide.

4. The method of claim 3, wherein said polypeptide is a membrane protein.

5. The method of claim 4, wherein said membrane protein is a G protein coupled receptor.

6. The method of claim 5, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

7. The method of claim 6, wherein said class A G protein coupled

receptor is m2 muscarinic cholinergic receptor.

8. The method of claim 1, wherein said at least one type of heterologous molecular linker includes a region for specifically binding the molecule-of-interest.

9. The method of claim 8, wherein the molecule-of-interest is a G protein coupled receptor and whereas said region for specifically binding the molecule-of-interest comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

10. The method of claim 9, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

11. The method of claim 9, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

12. The method of claim 9, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

13. The method of claim 9, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

14. The method of claim 9, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

15. The method of claim 14, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

16. The method of claim 8, wherein the molecule-of-interest includes a histidine tag and whereas said region for specifically binding the molecule-of-interest comprises a nickel ion or an antibody specific for said histidine tag.

17. The method of claim 8, wherein the molecule-of-interest includes core streptavidin and whereas said region for specifically binding the molecule-of-interest comprises a biotin moiety or a Strep-tag.

18. The method of claim 8, wherein the molecule-of-interest includes a biotin moiety or a Strep-tag and whereas said region for specifically binding the molecule-of-interest comprises core streptavidin.

19. The method of claim 1, wherein the molecule-of-interest is a G protein coupled receptor and whereas said at least one type of molecular linker comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

20. The method of claim 19, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370

of human beta-arrestin-1a.

21. The method of claim 9, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

22. The method of claim 19, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

23. The method of claim 19, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

24. The method of claim 19, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

25. The method of claim 24, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

26. The method of claim 1 wherein said at least one type of heterologous molecular linker includes at least two non-covalently bound subunits.

27. The method of claim 26, wherein said at least two non-covalently bound subunits comprise a first subunit comprising a homomultimerizing portion and a metal-binding portion, and a second subunit comprising a portion specifically binding the molecule-of-interest, and a portion specifically binding said first subunit.

28. The method of claim 26, wherein said at least two non-covalently bound subunits comprise a first subunit comprising a homomultimerizing portion and a portion specifically binding the molecule-of-interest, and a second subunit comprising a metal-binding portion, and a portion specifically binding said first subunit.

29. The method of claim 1, wherein said at least one type of heterologous molecular linker includes a molecule selected from the group consisting of a polycyclic molecule, a polydentate ligand, a macrobicyclic cryptand, a polypeptide and a metal.

30. The method of claim 1, wherein said at least one type of heterologous molecular linker comprises core streptavidin.

31. The method of claim 1, wherein said at least one type of heterologous molecular linker is selected so as to define the spatial positioning and orientation of said at least two molecules within said crystallizable molecular complex, thereby facilitating crystallization of the molecule-of-interest.

32. The method of claim 1, wherein said at least one type of heterologous molecular linker includes a hydrophilic region, said hydrophilic region being for facilitating crystallization of the molecule-of-interest.

33. The method of claim 1, wherein said at least one type of heterologous molecular linker includes a conformationally rigid region, said conformationally rigid region being for facilitating crystallization of the molecule-of-interest.

34. The method of claim 1, wherein said at least one type of heterologous molecular linker includes a metal-binding moiety capable of

specifically binding a metal atom, said metal atom being capable of facilitating crystallographic analysis of the crystal.

35. The method of claim 34, wherein said metal-binding moiety is a metal binding protein.

36. The method of claim 35, wherein said metal binding protein is metallothionein.

37. The method of claim 1, wherein said at least one type of heterologous molecular linker includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of said crystallizable molecular complex and/or of facilitating said interlinking at least two molecules of the molecule-of-interest.

38. The method of claim 37, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

39. The method of claim 1, wherein the molecule-of-interest includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of said crystallizable molecular complex, and/or of facilitating said interlinking at least two molecules of the molecule-of-interest.

40. The method of claim 39, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

41. The method of claim 1, wherein the molecule-of-interest includes a

metal-binding moiety capable of specifically binding a metal atom, said metal atom being capable of facilitating crystallographic analysis of the crystal.

42. The method of claim 41, wherein said metal-binding moiety is a metal binding protein.

43. The method of claim 42, wherein said metal binding protein is metallothionein.

44. A method of generating a crystal containing a polypeptide of interest, the method comprising:

(a) providing a molecule including the polypeptide of interest and a heterologous multimerization domain being capable of directing the homomultimerization of the polypeptide of interest;

(b) subjecting said molecule to homomultimerization-inducing conditions, thereby forming a crystallizable molecular complex; and

(c) subjecting said crystallizable molecular complex to crystallization-inducing conditions, thereby generating the crystal containing the polypeptide of interest.

45. The method of claim 44, wherein (a) and (b) are effected concomitantly.

46. The method of claim 44, wherein said heterologous multimerization domain is selected such that said crystallizable molecular complex formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

47. The method of claim 44, wherein said heterologous

multimerization domain includes a hydrophilic region, said hydrophilic region being for facilitating crystallization of the polypeptide of interest.

48. The method of claim 44, wherein said heterologous multimerization domain includes a conformationally rigid region, said conformationally rigid region being for facilitating crystallization of the polypeptide of interest.

49. The method of claim 44, wherein said heterologous multimerization domain is selected so as to define the spatial positioning and orientation of polypeptides of the polypeptide of interest within said crystallizable molecular complex, thereby facilitating crystallization of the polypeptide of interest.

50. The method of claim 44, wherein said heterologous multimerization domain comprises core streptavidin.

51. The method of claim 44, wherein the polypeptide of interest is a G protein coupled receptor and whereas said heterologous multimerization domain comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

52. The method of claim 51, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

53. The method of claim 52, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

54. The method of claim 51, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

55. The method of claim 51, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

56. The method of claim 51, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

57. The method of claim 56, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

58. The method of claim 44, wherein the polypeptide of interest includes a histidine tag and whereas said heterologous multimerization domain comprises a nickel ion or an antibody specific for said histidine tag.

59. The method of claim 44, wherein the polypeptide of interest includes core streptavidin and whereas said heterologous multimerization domain comprises a biotin moiety or a Strep-tag.

60. The method of claim 44, wherein the polypeptide of interest includes a biotin moiety or a Strep-tag and whereas said heterologous multimerization domain comprises core streptavidin.

61. The method of claim 44, wherein the polypeptide of interest and said heterologous multimerization domain are interlinked via a molecular linker.

62. The method of claim 61, wherein at least one of said heterologous multimerization domain and said molecular linker include a hydrophilic region, said hydrophilic region being for facilitating crystallization of the polypeptide of interest.

63. The method of claim 61, wherein at least one of said heterologous multimerization domain and said molecular linker include a conformationally rigid region, said conformationally rigid region being for facilitating crystallization of the polypeptide of interest.

64. The method of claim 61, wherein at least one of said heterologous multimerization domain and said molecular linker is selected so as to define the spatial positioning and orientation of polypeptides of the polypeptide of interest within said crystallizable molecular complex, thereby facilitating crystallization of the polypeptide of interest.

65. The method of claim 61, wherein said at least one molecular linker includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of said crystallizable molecular complex, and/or of facilitating said homomultimerization of the polypeptide of interest.

66. The method of claim 65, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

67. The method of claim 44, wherein the polypeptide of interest

includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of said crystallizable molecular complex, and/or of facilitating said homomultimerization of the polypeptide of interest.

68. The method of claim 67, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

69. The method of claim 44, wherein said molecule includes a metal-binding moiety capable of specifically binding a metal atom, said metal atom being capable of facilitating crystallographic analysis of the crystal.

70. The method of claim 69, wherein said metal-binding moiety is a metal binding protein.

71. The method of claim 70, wherein said metal binding protein is metallothionein.

72. The method of claim 44, wherein the polypeptide of interest is a membrane protein.

73. The method of claim 72, wherein said membrane protein is a G protein coupled receptor.

74. The method of claim 73, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

75. The method of claim 74, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

76. The method of claim 44, wherein the polypeptide of interest includes a metal-binding moiety capable of specifically binding a metal atom, said metal atom being capable of facilitating crystallographic analysis of the crystal.

77. The method of claim 70, wherein said metal binding moiety is metallothionein.

78. A composition-of-matter comprising at least two molecules of a molecule-of-interest interlinked via a heterologous molecular linker, wherein said heterologous molecular linker is selected so as to define the relative spatial positioning and orientation of said at least two molecules within the composition-of-matter, thereby facilitating formation of a crystal therefrom under crystallization-inducing conditions.

79. The composition-of-matter of claim 78, wherein the molecule-of-interest is a polypeptide.

80. The composition-of-matter of claim 79, wherein said polypeptide is a membrane protein.

81. The composition-of-matter of claim 80, wherein said membrane protein is a G protein coupled receptor.

82. The composition-of-matter of claim 81, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

83. The composition-of-matter of claim 82, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

84. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes at least one region capable of specifically binding said molecule-of-interest.

85. The composition-of-matter of claim 84, wherein said molecule-of-interest is a G protein coupled receptor and whereas said at least one region capable of specifically binding said molecule-of-interest is a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

86. The composition-of-matter of claim 85, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

87. The composition-of-matter of claim 86, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

88. The composition-of-matter of claim 85, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

89. The composition-of-matter of claim 85, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

90. The composition-of-matter of claim 85, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

91. The composition-of-matter of claim 90, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

92. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes a molecule selected from the group consisting of a polycyclic molecule, a polydentate ligand, a macrobicyclic cryptand, a polypeptide and a metal.

93. The composition-of-matter of claim 78, wherein said molecule-of-interest is a G protein coupled receptor and whereas said heterologous molecular linker comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

94. The composition-of-matter of claim 93, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

95. The composition-of-matter of claim 94, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

96. The composition-of-matter of claim 93, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin

is a mutation to a serine or threonine residue.

97. The composition-of-matter of claim 93, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

98. The composition-of-matter of claim 93, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

99. The composition-of-matter of claim 98, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

100. The composition-of-matter of claim 78, wherein said heterologous molecular linker comprises core streptavidin.

101. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes at least two non-covalently bound subunits.

102. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes a hydrophilic region, said hydrophilic region being for facilitating crystallization of said molecule-of-interest.

103. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes a conformationally rigid region, said conformationally rigid region being for facilitating crystallization of said molecule-of-interest.

104. The composition-of-matter of claim 78, wherein said heterologous molecular linker is selected such that the composition-of-matter is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

105. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes a metal-binding moiety capable of specifically binding a metal atom, said metal atom being capable of facilitating crystallographic analysis of the crystal.

106. The composition-of-matter of claim 105, wherein said metal-binding moiety is a metal-binding protein.

107. The composition-of-matter of claim 106, wherein said metal binding protein is metallothionein.

108. The composition-of-matter of claim 78, wherein said heterologous molecular linker includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of the crystallizable composition-of-matter, and/or of facilitating said interlinking of said at least two molecules of a molecule-of-interest.

109. The composition-of-matter of claim 78, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

110. The composition-of-matter of claim 78, wherein said molecule-of-interest includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of the composition-of-matter, and/or of facilitating said interlinking of said at least two molecules of a molecule-of-interest.

111. The composition-of-matter of claim 110, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

112. The composition-of-matter of claim 78, wherein said molecule-of-interest includes a metal-binding moiety capable of specifically binding a metal atom, said metal atom being capable of facilitating crystallographic analysis of the crystal.

113. The composition-of-matter of claim 112, wherein said metal-binding moiety is a metal binding protein.

114. The composition-of-matter of claim 113, wherein said metal-binding protein is metallothionein.

115. A nucleic acid construct comprising a polynucleotide segment encoding a chimeric polypeptide including:

(a) a first polypeptide region being capable of specifically binding a molecule-of-interest; and

(b) a second polypeptide region being capable of specifically binding a metal atom.

116. The nucleic acid construct of claim 115, wherein said molecule-of-interest is a G protein coupled receptor and whereas said chimeric polypeptide comprises SEQ ID NO: 5 or SEQ ID NO: 6.

117. The nucleic acid construct of claim 116, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

118. The nucleic acid construct of claim 117, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

119. The nucleic acid construct of claim 115, wherein said molecule-of-interest is a G protein coupled receptor and whereas said first polypeptide region

comprises a molecule selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

120. The nucleic acid construct of claim 119, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

121. The nucleic acid construct of claim 120, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

122. The nucleic acid construct of claim 119, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

123. The nucleic acid construct of claim 119, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

124. The nucleic acid construct of claim 119, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

125. The nucleic acid construct of claim 124, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

126. The nucleic acid construct of claim 115, wherein the molecule-of-

interest is a polypeptide.

127. The nucleic acid construct of claim 126, wherein said polypeptide is a membrane protein.

128. The nucleic acid construct of claim 127, wherein said membrane protein is a G protein coupled receptor.

129. The nucleic acid construct of claim 128, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

130. The nucleic acid construct of claim 129, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

131. The nucleic acid construct of claim 115, wherein said second polypeptide region is metallothionein.

132. The nucleic acid construct of claim 115, wherein said chimeric polypeptide is selected such that when combined with molecules of said molecule-of-interest under suitable conditions, said chimeric polypeptide and said molecules form a crystallizable molecular complex which is capable of forming a crystal containing said molecule-of-interest when subjected to crystallization-inducing conditions.

133. The nucleic acid construct of claim 115, wherein said chimeric polypeptide is selected such that when combined with molecules of said molecule-of-interest and said metal atom under suitable conditions, said chimeric polypeptide and said molecules form a crystallizable molecular complex which is capable of forming a crystal containing said molecule-of-interest when subjected to crystallization-inducing conditions.

134. The nucleic acid construct of claim 132, wherein said metal atom facilitates crystallographic analysis of said crystal.

135. The nucleic acid construct of claim 132, wherein said chimeric polypeptide includes a hydrophilic region, said hydrophilic region being for facilitating crystallization of said molecule-of-interest.

136. The nucleic acid construct of claim 132, wherein said chimeric polypeptide includes a conformationally rigid region, said conformationally rigid region being for facilitating crystallization of said molecule-of-interest.

137. The nucleic acid construct of claim 132, wherein said chimeric polypeptide is selected so as to define the spatial positioning and orientation of said molecule-of-interest within said crystallizable molecular complex, thereby facilitating crystallization of said molecule-of-interest.

138. The nucleic acid construct of claim 132, wherein said chimeric polypeptide is selected such that said crystallizable molecular complex formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

139. The nucleic acid construct of claim 132, wherein said chimeric polypeptide further includes a polypeptide region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of said crystallizable molecular complex, and/or of facilitating said binding of a molecule-of-interest.

140. The nucleic acid construct of claim 139, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

141. A nucleic acid construct comprising a polynucleotide segment encoding a chimeric polypeptide including:

(a) a first polypeptide region being capable of specifically binding a molecule-of-interest;

(b) a second polypeptide region being capable of homomultimerization into a complex of defined geometry; and

(c) a third polypeptide region being capable of specifically binding a metal atom.

142. The nucleic acid construct of claim 141, wherein said molecule-of-interest is a G protein coupled receptor and whereas said first polypeptide region is selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, SEQ ID NO: 3, and SEQ ID NO: 4.

143. The nucleic acid construct of claim 142, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

144. The nucleic acid construct of claim 143, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

145. The nucleic acid construct of claim 142, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

146. The nucleic acid construct of claim 9, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

147. The nucleic acid construct of claim 142, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

148. The nucleic acid construct of claim 147, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

149. The nucleic acid construct of claim 141, wherein said second polypeptide region comprises core streptavidin.

150. The nucleic acid construct of claim 141, wherein said molecule-of-interest is a G protein coupled receptor and whereas said chimeric polypeptide comprises SEQ ID NO: 5 or SEQ ID NO: 6.

151. The nucleic acid construct of claim 150, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

152. The nucleic acid construct of claim 151, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

153. The nucleic acid construct of claim 141, wherein said third polypeptide region comprises metallothionein.

154. The nucleic acid construct of claim 141, wherein the molecule-of-interest is a polypeptide.

155. The nucleic acid construct of claim 154, wherein said polypeptide

is a membrane protein.

156. The nucleic acid construct of claim 155, wherein said membrane protein is a G protein coupled receptor.

157. The nucleic acid construct of claim 156, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

158. The nucleic acid construct of claim 157, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

159. The nucleic acid construct of claim 141, wherein said chimeric polypeptide is selected such that when combined with molecules of said molecule-of-interest, said chimeric polypeptide and said molecules form a crystallizable molecular complex of defined geometry which is capable of forming a crystal containing said molecule-of-interest when subjected to crystallization-inducing conditions.

160. The nucleic acid construct of claim 159, wherein said chimeric polypeptide includes a hydrophilic region, said hydrophilic region being for facilitating crystallization of said molecule-of-interest.

161. The nucleic acid construct of claim 159, wherein said chimeric polypeptide includes a conformationally rigid region, said conformationally rigid region being for facilitating crystallization of said molecule-of-interest.

162. The nucleic acid construct of claim 159, wherein said chimeric polypeptide is selected so as to define the spatial positioning and orientation of molecules of said molecule-of-interest within said crystallizable molecular complex, thereby facilitating crystallization of said molecule-of-interest.

163. The nucleic acid construct of claim 159, wherein said chimeric polypeptide is selected such that said crystallizable molecular complex of defined geometry formed is capable of generating a crystal selected from the group consisting of a 2D crystal, a helical crystal and a 3D crystal.

164. The nucleic acid construct of claim 159, wherein said metal atom facilitates crystallographic analysis of said molecule-of-interest contained in said crystal.

165. The nucleic acid construct of claim 159, wherein said chimeric polypeptide further includes a polypeptide region being capable of functioning as a purification tag, said purification tag being capable of facilitating purification of said crystallizable molecular complex, and/or of facilitating said binding of a molecule-of-interest.

166. The nucleic acid construct of claim 165, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, and core streptavidin.

167. A method of purifying a G protein coupled receptor from a sample containing the G protein coupled receptor, the method comprising subjecting the sample to affinity chromatography using an affinity ligand selected from the group consisting of at least a portion of an arrestin molecule, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin, at least a portion of an arrestin molecule having a mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin, a molecule defined by SEQ ID NO: 3, and a molecule defined by SEQ ID NO: 4, thereby purifying the G protein coupled receptor.

168. The method of claim 167, wherein said at least a portion of an arrestin molecule is homologous to amino acid residues 11 to 190, or 11 to 370 of human beta-arrestin-1a.

169. The method of claim 168, wherein said at least a portion of an arrestin molecule comprises a G protein coupled receptor-binding domain of said arrestin molecule.

170. The method of claim 167, wherein said mutation at an amino acid residue position corresponding to position 90 in bovine visual arrestin is a mutation to a serine or threonine residue.

171. The method of claim 167, wherein said mutation at an amino acid residue position corresponding to position 175 in bovine visual arrestin is a mutation to a glutamic acid or an asparagine residue.

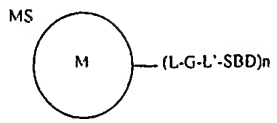
172. The method of claim 167, wherein said G protein coupled receptor is rhodopsin or is a class A G protein coupled receptor.

173. The method of claim 172, wherein said class A G protein coupled receptor is m2 muscarinic cholinergic receptor.

174. The method of claim 167, wherein said affinity ligand includes a region being capable of functioning as a purification tag, said purification tag being capable of facilitating attachment of said affinity ligand to an affinity chromatography matrix.

175. The method of claim 174, wherein said region being capable of functioning as a purification tag is selected from the group consisting of a T7 tag, a histidine tag, a Strep-tag, core streptavidin, and biotin.

Fig. 1a



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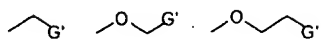

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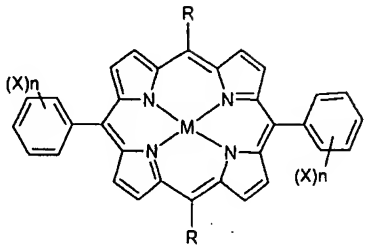

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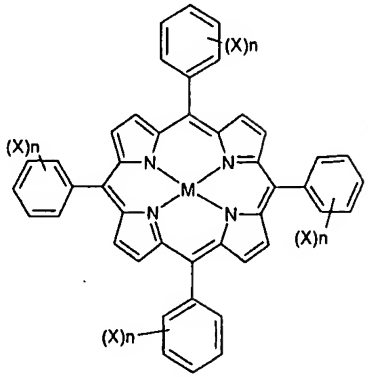

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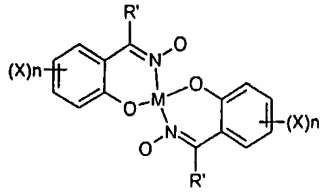

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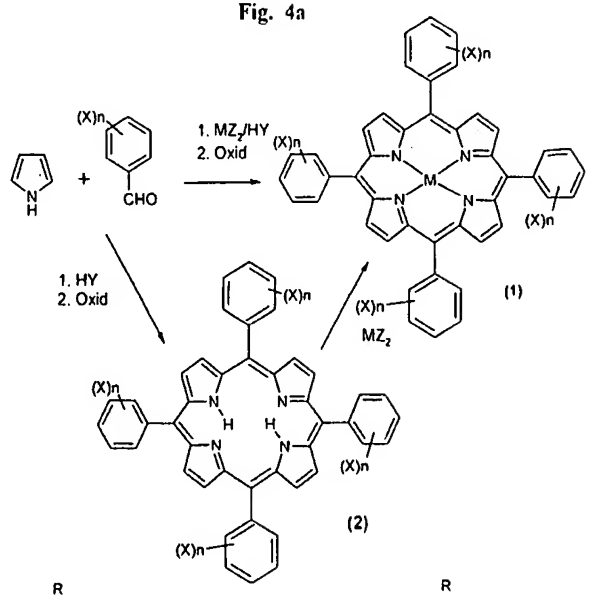

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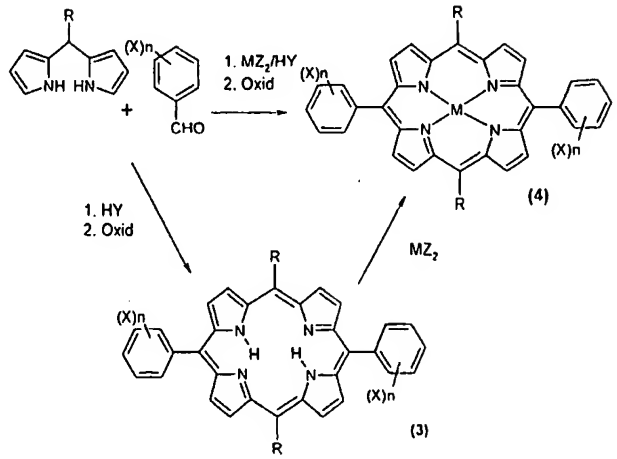

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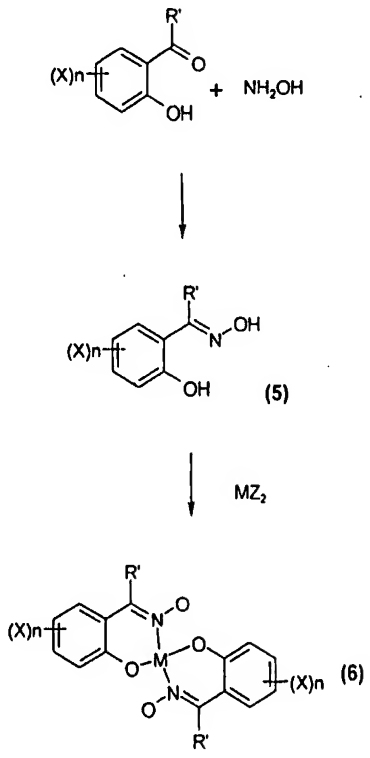

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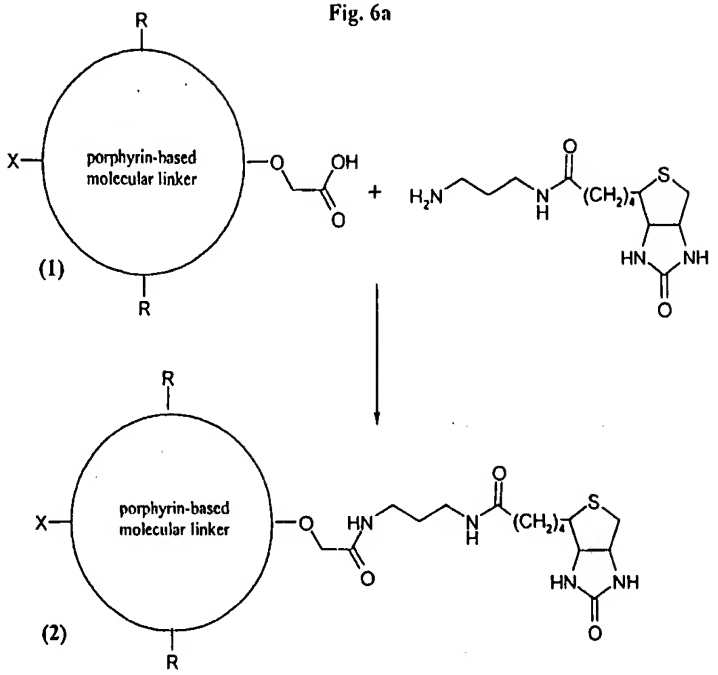

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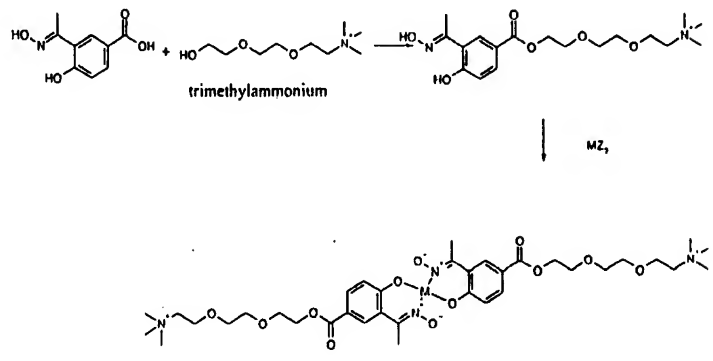

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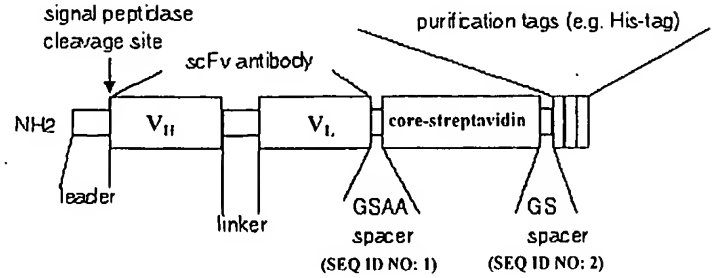

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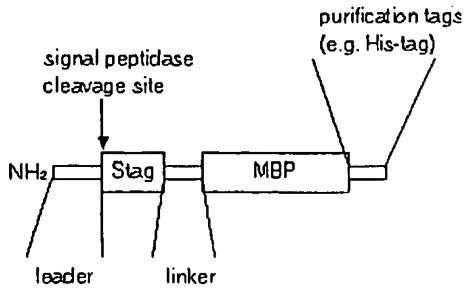

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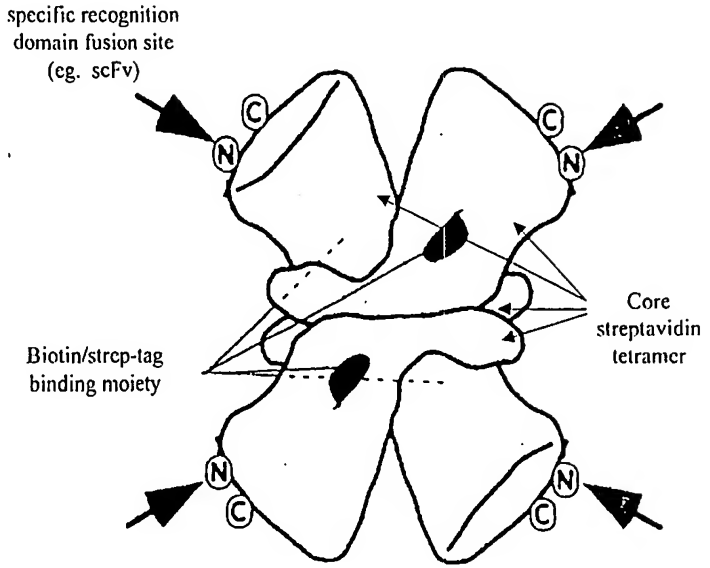

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Fig. 9b

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Fig. 10b

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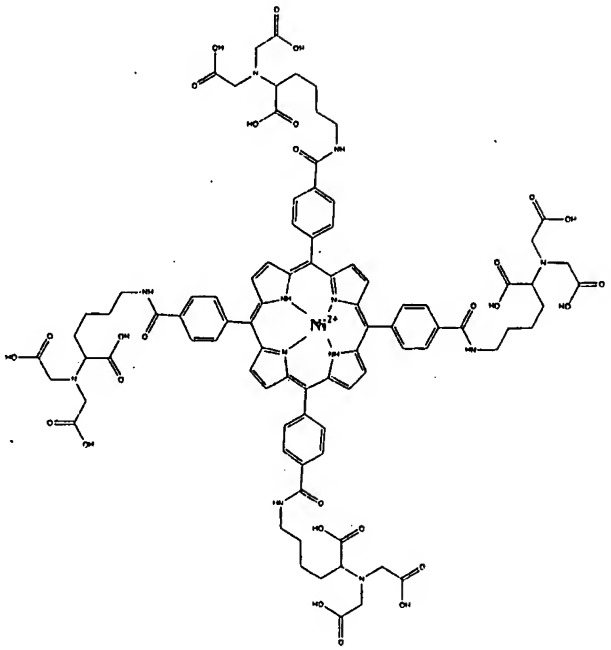
Fig. 11



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Thin Solid Films, 244 (1994) 789–793

Self-assembly of avidin and streptavidin with multifunctional biotin molecules

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Institute of Molecular and Biomolecular Electronics, University of Wales, Dean Street, Bangor, Gwynedd LL57 1UT (UK)

Abstract

We report the synthesis of a water-soluble tetrafunctional biotin ligand based on the porphyrin moiety and its behaviour at the air–water interface. The addition of streptavidin or avidin to the subphase is shown to cause a significant expansion of the isotherm of the tetrabiotinylated ligand, indicating a strong interaction between the protein and the ligand. The addition of inactive protein to the subphase caused no such change from which it is deduced that non-specific interactions could not have been responsible for the effects observed with the active proteins. Supplementary experiments using column chromatography provide evidence for the formation of a high molecular weight polymer when the tetrabiotinylated ligand and active protein are mixed.

1. Introduction

Harnessing the self-assembling property of functional molecular units is perceived to be a key technique for the fabrication of electronic circuits on a molecular scale and much progress has been made in this direction as a result of advances in synthetic chemistry and in protein chemistry. The ability of biomacromolecules to recognize specific molecular ligands provides an attractive approach to the self-assembly of molecular systems [1] and, in particular, specific molecular recognition based on non-covalent binding interactions [2, 3] has enormous potential. By synthesizing a series of homologues which incorporate ligands that form one part of a binding pair it becomes feasible to assemble systems capable of being exploited in molecular electronics [4].

Already we have reported an investigation of affinity polymerization as a self-assembly technique. The method is based on the strong affinity of the proteins avidin and streptavidin for their complementary ligand, biotin, and has enabled us to fabricate multilayers of protein on a solid substrate [5]. Recently, we have investigated the polymerization of avidin and streptavidin using a variety of bisbiotin ligands based on aromatic molecules [6].

Blankenburg *et al.* [7] have shown that streptavidin forms two-dimensional (2D) crystalline aggregates at the air–water interface when added to a subphase supporting a monolayer of biotinlipid. Although we are also interested in forming 2D aggregates our objective is to do so by interlinking proteins within a layer. In such a network, each molecular element would be physically connected to adjacent elements, thus, in principle at least, facilitating the transport of signals from one element to the next. Since the binding pockets of avidin and streptavidin are arranged in pairs on opposite sides of the molecule, the formation of 2D networks with these proteins requires the synthesis of a tetrabiotinylated ligand (Fig. 1). To achieve this goal, the porphyrin molecule was chosen to be the central moiety of the tetrafunctionalized ligand since it is easily modified chemically to allow the incorporation of four biotin moieties. Furthermore, porphyrin has novel and interesting physical properties in its own right, *e.g.* photoconductivity, which may make the protein–porphyrin structure an interesting model system for the study of electron tranfer processes [8].

In this paper we report (i) the synthesis of a water-soluble tetrabiotin ligand, (ii) its monolayer-forming properties at the air–water interface and (iii) its interaction with avidin and streptavidin.

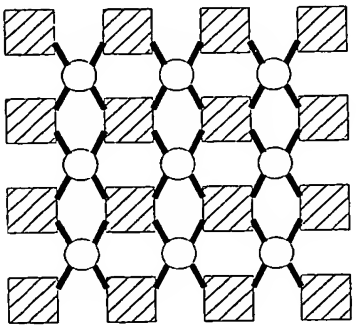

Fig. 1. Schematic diagram of a 2D network formed by the specific binding of a tetrameric protein such as streptavidin with a tetrafunctionalized ligand.

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SSDI 0040-6090(93)04109-6

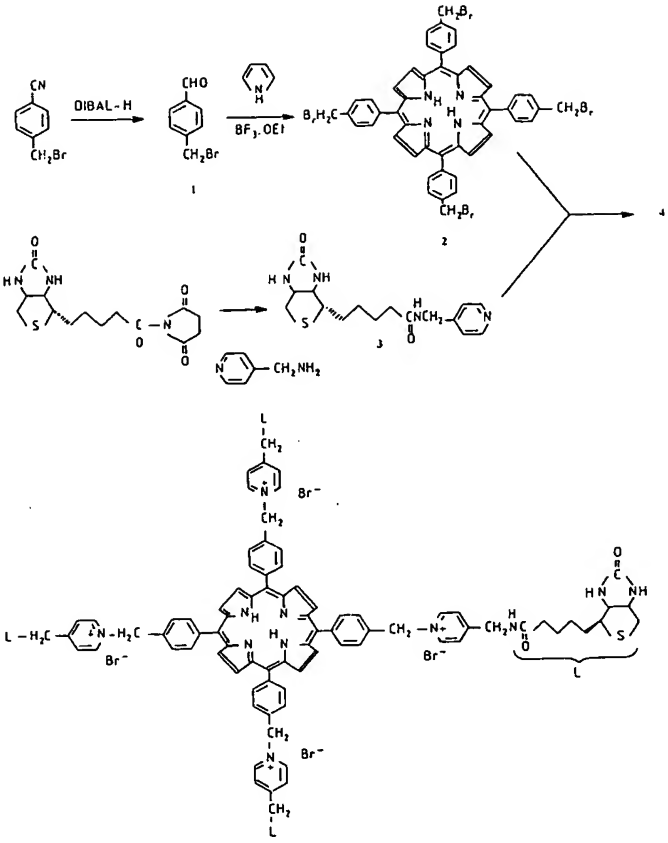
2. Experimental details

2.1. Synthesis of a tetrafunctional biotin ligand

Our previous work [6] and that of Green *et al.* [9] have shown that the length and flexibility of the binding ligand are crucial factors in determining the stability of avidin and streptavidin polymers. On the basis of these considerations and noting that a water-soluble tetrabiotin would enable polymers to be formed in aqueous solution, the target molecule chosen was 5,10,15,20-tetrakis {α-[4-(biotinylamidomethyl) pyridinium bromide]-*p*-tolyl} porphyrin (TBPP) (**4**). In this molecule the distance between the carbonyl groups of two diammetrically opposed biotin ligands has been determined from molecular modelling to be over 3 nm. This should be sufficiently long so that four proteins can be immobilized on a single tetrabiotin molecule without steric hindrance. While the basic structure of the porphyrin is a rigid plane, the sp^3 carbon atoms attached to the benzene and pyridine rings impart some flexibility to the terminal biotin moieties.

The synthetic strategy involved substituting *p*-bromomethyl benzene units into the 5, 10, 15, and 20 positions in the porphyrin. Subsequently, a pyridinium unit in the form of a pyridinium bromide salt was connected to the bromomethyl moiety. The biotin molecule was then attached to the *para* position of the pyridine ring in order to achieve the maximum distance between diammetrically opposed biotin ligands.

α-Bromo-*p*-tolunitrile was chosen as the starting material for synthesizing the porphyrin skeleton. The cyano group was converted to aldehyde by diisobutylaluminium hydride (DIBAL-H) with a 78% yield (Scheme 1). The porphyrin was synthesized by the reaction between aldehyde (**1**) and pyrrole with catalytic trifluoride etherate (BF_3OEt) in dry $CHCl_3$ following the method of Lindsey *et al.* [10]. The reaction mixture was purified by basic alumina column chromatograpy and porphyrin tetrabromide (5,10,15,20-tetrakis (α-bromo-*p*-tolyl) porphyrin (**2**) was obtained as fine purfied crystals (yield, 37%). The modification of biotin was performed by introducing the pyridinium group into the carboxylic acid chain. The reaction between biotinyl-hydroxysuccinimide (BNHS) and 4-aminomethylpyridine with silica gel purification resulted in colourless fine crystals of 3-(biotinyl amido)pyridine (**3**) (yield, 88%). The final stage of the reaction was carried out between the porphyrin **2** and biotin ester **3** in dry dimethylformamide at 60 °C [11]. Temperature control of the reaction mixture was essential because above 70 °C an insoluble polymer formed. Attempts at purifying the final product by recrystallization were unsuccessful. However, gel filtration using sephadex G-25 columns was found to be a simple and efficient technique for purifying the crude mixture. The



Scheme 1.

tetrabiotin porphyrin **4** was obtained as fine purple crystals (yield, 20%). The compound gave satisfactory spectroscopic, analytical and mass spectral data. The Soret band of the biotinylated porphyrin dissolved in pure water was at 415.5 nm compared with 420.5 nm for the tetrabromoporphyrin precursor dissolved in chloroform.

2.2. Preparation of monolayer

The monolayer-forming properties of TBPP were investigated in a polytetrafluoroethylene trough of the sliding barrier type located on an antivibration table housed in a class 2 semiconductor clean-room. Pure water for washing and for the trough was obtained from a Millipore Milli-RO60 reverse osmosis cartridge coupled to a Super Q system comprising ion exchange, Organex and 0.2 μm filter cartridges. The surface pressure was monitored with a Wilhelmy plate and electrobalance to an accuracy of 0.1 mN m^{-1}. For all the experiments reported here the subphase was 0.25 M NaCl held at a constant temperature of 28 °C. The presence of the salt was intended to reduce the possibility of non-specific binding of protein to the monolayer in subsequent experiments. The spreading solution was prepared by firstly dissolving 1 mg of TBPP in

1 ml of ultrapure water and then mixing 30 µl of this solution with 0.4 ml of a methanol–chloroform mixture (methanol:chloroform = 1:1). Pressure–area isotherms were obtained by spreading an aliquot of the final solution on the subphase surface and waiting for about 30 min before compression. The isotherms were obtained at a compression rate of 0.018 nm^2 $molecule^{-1}$ s^{-1}.

Preliminary investigations revealed very quickly that the water solubility of TBPP was too great to allow stable monolayers to form. To reduce the loss of material to the subphase, an ionic complex was formed between TBPP and a long-chain alkanoic salt which provided a hydrophobic anion to replace the Br^- counter-anion. Ionic interaction between the insoluble, hydrophobic anion and the TBPP cation was expected to improve the stability of the TBPP monolayer.

A similar strategy was adopted by Barraud and coworkers [12–14] who utilized the chemical reaction between a pyridinium salt containing porphyrin and a fatty acid such as stearic acid to form an ionic complex that was stable at the air–water interface. In the present work, sodium octadecyl sulphate (ODS) was chosen as the anchoring molecule for TBPP. The complex was formed by mixing 20 µl of ODS (1 mg in 1 ml of a methanol–chloroform mixture (methanol:chloroform = 2:8)) with 30 µl of the aqueous TBPP solution prior to final dilution in the methanol–chloroform spreading solvent (methanol:chloroform = 1:1) as above. The ionic complex formed spontaneously in the resulting solution in which the TBPP:ODS mole ratio was 1:4. Aliquots of this mixutre were then spread on the subphase surface and the pressure isotherm obtained under the same conditions as before.

2.3. *Immobilization of proteins to monolayer*

Immobilization of streptavidin (Vector Laboratories Ltd., Peterborough, UK), avidin (type D from Vector Laboratories Ltd.) and succinylated avidin (Sigma Chemicals, St. Louis, MO) was carried out following the procedures reported by Blankenburg *et al.* [7]. A solution composed of 0.5 mg of protein in 3 ml of 0.25 M NaCl was prepared and, using a microsyringe, injected into the subphase at several positions beneath an expanded TBPP–ODS monolayer on the subphase surface. The monolayer was then left to incubate for 2 h at 29 °C.

3. Results and discussion

3.1. *Formation of the monolayer*

Isotherms obtained for pure TBPP showed clear evidence of dissolution into the subphase. The onset area for pressure rise was about 2.8 nm^2 per complex and at the low area limit of the trough, although the surface pressure had risen to 27 mN m^{-1}, the area per complex had decreased to about 0.8 nm^2. This compares with an estimated area of 2.2–2.4 nm^2 for the tetrapyridiniumporphyrin moiety based on the assumption that the cross-shaped molecule occupied a square area with side equal to the distance between pyridinium moieties. The shift in the isotherm to even smaller areas for subsequent compressions coupled with its known high water solubility is strong evidence that TBPP dissolves into the subphase during compression. When complexed with ODS the isotherm (full curves in Figs. 2 and 3) was more expanded; the area per complex at the onset of pressure rise was equal to 6.75 nm^2 and decreased to only 2.3 nm^2 just prior to collapse at a surface pressure of about 40 mN m^{-1}. Since the area at collapse is close to that expected for the tetrapyridiniumporphyrin moiety, we may assume that this moiety lies flat on the water surface and that the biotin moieties are directed either into the water or into the air. The ODS anions presumably occupy the spaces between adjacent TBPP molecules where they can remain close to the oppositely charged pyridinium groups. So long as the monolayer was not compressed to collapse, the expansion isotherm followed that obtained during the first compression. However, for a monolayer compressed beyond collapse, significant hysteresis was observed when the barriers were opened. Nevertheless, isotherms obtained during subsequent compressions of the monolayer were identical with that obtained initially, confirming therefore that no material is lost from the monolayer during collapse.

3.2. *The immobilization of proteins*

The effect of introducing streptavidin into the subphase supporting a TBPP–ODS monolayer is shown in Fig. 2 (chain curve). After incubation for 2 h a considerable expansion of the monolayer occurred which we presumed to be caused by protein binding to the monolayer. To confirm that binding was specific, the experiment was repeated with inactive strepavidin; the latter was prepared by adding sufficient biotin to an aliquot of the streptavidin solution to block all four binding sites in the protein. The broken curve in Fig. 2 shows that inactive streptavidin has a negligible effect on the isotherm of TBPP–ODS, a result which confirms that little nonspecific binding of protein to monolayer occurred. This is not surprising since the particular streptavidin used had a pI of about 7 and, with the subphase pH held at 6.5, the net charge on the protein would have been low. Furthermore, the NaCl subphase would have further decreased any charge interactions between protein and monolayer. The TBPP–ODS–protein layer was stable under compression, the area decreasing by only 0.3% min^{-1} at a pressure of 30 mN m^{-1}. This is sufficiently stable to allow deposition onto solid supports.

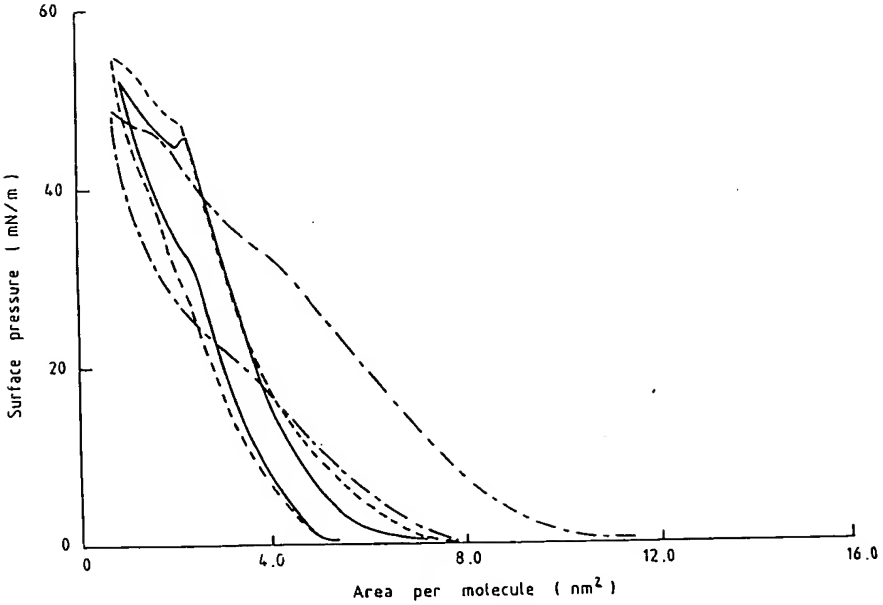


Fig. 2. Pressure–area isotherms for TBPP-ODS before (——) and after addition of active (– – –) and inactive (– – –) streptavidin to the subphase. The isotherms showing the effects of protein addition were obtained after incubating the monolayer for 2 h at 29 °C.

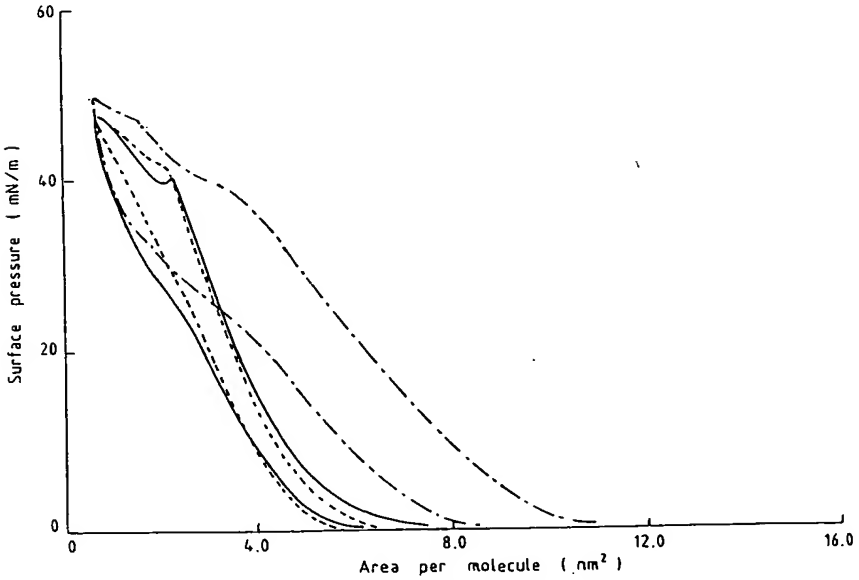


Fig. 3. Pressure–area isotherms for TBPP-ODS before (——) and after addition of active (– – –) and inactive (– – –) avidin to the subphase. The isotherms showing the effects of protein addition were obtained after incubating the monolayer for 2 h at 29 °C.

The results of similar experiments with avidin are shown in Fig. 3. As can be seen, the behaviour is virtually identical with that observed with streptavidin. Normally, avidin obtained from commercial sources is expected to show a high degree of non-specific binding either because of its high pI (10–11) or because of the presence of sugar residues. The avidin used in this work, however, was described as having a low-degree of non-specific adsorption. Using isoelectric focusing gels we have already shown [15] that the isoelectric point of this particular protein is in the pH range 7.5–8 so at the pH of the experiment the protein will be only weakly charged. Thus non-specific binding is expected to be low, consistent with the observation in Fig. 3. Interestingly, an inactive succinylated avidin (pI about 4) did cause slight expansion of the monolayer, indicating the

occurrence of non-specific binding in this case. It seems therefore that non-specific binding is related more to the charged state of the proteins rather than to the presence or otherwise of sugar residues in the protein.

In a supplementary experiment in which active protein was mixed with an excess of TBPP–ODS a reddish-coloured precipitate formed which would not pass through a Sephadex G-100 gel filtration column, suggesting that either a highly cross-linked polymer or perhaps a gel had been formed. In previous work with bisbiotin ligands [6], linear polymers, oligomers and protein monomers all passed through the column.

4. Conclusions

A tetrabiotinylated ligand based on the porphyrin moiety has been synthesized and its monolayer behaviour at the air-water interface investigated. The addition of active streptavidin or avidin to the subphase caused, after a 2 h incubation, a significant expansion in the pressure isotherm of the TBPP–ODS, suggesting a strong interaction between the protein and the monolayer. That a specific interaction was occurring was confirmed by the negligible change in the isotherm after addition of inactive protein to the subphase. The lack of non-specific binding by the inactive avidin was attributed to its low charge state at the pH of the experiment. Although we have no direct evidence for polymer formation at the air–water interface, we have shown that mixing protein with an excess of TBPP–ODS results in a reddish precipitate which does not pass through a Sephadex gel filtration column. The absence of protein monomer passing through the column led to the conclusion that the precipitate was either a crosslinked polymer or a gel.

Acknowledgments

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References

1 J. S. Lindsey, *New J. Chem.*, *15* (1991) 153.
2 D. J. Cram, *Angew. Chem., Int. Edn. Engl.*, *27* (1988) 1009.
3 F. Vogtle and E. Weber (eds.), *Host Guest Complex Chemistry. Macrocycles*, Springer, Berlin, 1985.
4 D. M. Taylor, H. Morgan and C. D'Silva, *J. Colloid Interface Sci.*, *144* (1991) 53.
5 D. M. Taylor, H. Morgan, C. D'Silva and H. Fukushima, *Thin Solid Films*, *210–211* (1992) 773.
6 H. Morgan, H. Fukushima and D. M. Taylor, *J. Poly. Sci. A*, in press.
7 R. Blankenburg, P. Meller, H. Ringsdorf and C. Salesse, *Biochemistry*, *26* (1989) 8221.
8 M. R. Wasielewski, *Chem. Rev.*, *92* (1992) 435.
9 N. M. Green, L. Koneczny, K. J. Toms and R. C. Valentine, *Biochem. J.*, *125* (1971) 781.
10 J. S. Lindsey, I. C. Scheriman, H. C. Hsu, P. C. Kearney and A. M. Marguerettaz, *J. Org. Chem.*, *52* (1987) 827.
11 L. R. Milgrom, *J. Chem. Soc., Perkin. Trans.*, (1983) 2535.
12 A. Ruadel-Teixier and A. Barraud, *Thin Solid Films*, *99* (1983) 33.
13 S. Palacin, P. Leiseur, L. Stefanelli and A. Barraud, *Thin Solid Films*, *83* (1988) 83.
14 F. I. Parteu, S. Palacin, A. Ruadel-Teixier and A. Barraud, *Thin Solid Films*, *210–211* (1992) 769.
15 D. M. Taylor, H. Morgan and C. D'Silva, *J. Phys. D*, *24* (1991) 1443.

Synthesis and Monolayer Behavior of a Tetrabiotinylated Porphyrin Ligand

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The synthesis of a tetrabiotinylated porphyrin ligand, 5,10,15,20-tetrakis{α-[4-(biotinylamidomethyl)-pyridinium bromide]-*p*-tolyl}porphyrin (TBPP), is described and its monolayer behavior at the air–water interface investigated. Monolayers of the pure salt were found to be unstable. The effects of complexing with different mole ratios of sodium octadecyl sulfate (ODS) were studied. The TBPP–ODS (1:4) complex gave stable isotherms from which it was deduced that porphyrin molecules lay flat on the water surface, with biotin moieties pointing into the subphase. From STM images it was deduced that the high deposition ratio (~1.5) during vertical dipping probably arose from a spontanous self-assembly of the complex into rodlike stacks. Fluorescence microscopy showed that, in the "gaseous" phase, the complex assembled into a network of associated porphyrin molecules surrounding circular voids of different size. Addition of active avidin or streptavidin caused a significant expansion of the isotherm that was accompanied by the appearance of a domain-like structure in the monolayer. That no such changes were observed with inactive proteins confirms the specific nature of the interactions being investigated.

1. Introduction

Avidin and streptavidin are highly stable, robust proteins. These properties coupled to their high affinity for biotin have already led to the widespread use of the streptavidin/biotin complex in a range of technological applications such as immunoassays, biosensors, affinity chromatography, and directed drug or isotope delivery.[1] Our interest in this protein/ligand couple stems from our attempts to form 2-D protein arrays as possible templates for the fabrication of molecular electronic systems. The approach is based on the developments in microelectronic systems where very large scale integrated (VLSI) logic arrays and memories are essentially 2-D arrays of repeating elements. Using proteins as the basic components in the array, it is our intention to incorporate functionality into the binding ligand and, ultimately, into the protein, thus bringing a molecular electronic system closer to reality.

We have already reported on the synthesis of aromatic bisbiotin ligands and have investigated their ability to form linear polymers with avidin and streptavidin.[2] The basis of polymer formation is the strong affinity ($K_d \sim 10^{-15}$ M) between these proteins and their complementary ligand, biotin. Because the binding pockets are arranged in pairs on opposite sides of the molecule, bisbiotin ligands form linear chains of interlinked protein molecules despite the tetrameric structure of these proteins.[3-8]

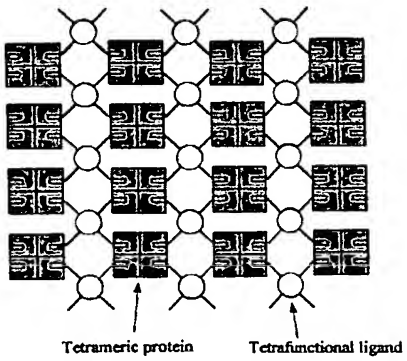

Figure 1. A two-dimensional array based on the binding between a tetrafunctionalized ligand and a tetrameric protein.

Therefore, to form a 2-D array such as that shown in Figure 1 a tetrafunctionalized ligand is essential. Preferably, the ligand must be of a square-planar rather than a tetrahedral structure so as to maintain the planarity of the array. The side chains must be sufficiently long and flexible to allow four proteins to be bound to one ligand without steric hindrance. Ideally, the ligand should be water soluble thus enabling the polymerization of protein to take place in an aqueous medium so as to avoid denaturing the protein. An advantage of this approach is that, even if the tetrahedral structure of the protein may militate against the formation of the network in Figure 1, there still exists the possibility of forming a 2-D network in which only two of the protein binding pockets (adjacent or opposed) link the planar tetrafunctionalized ligands. In this case, the mole fraction of ligand in the network will be reduced to a half of that in Figure 1.

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(1) Wilchek, M.; Bayer, E. A. *Anal. Biochem.* **1988**, *171*, 1.
(2) Morgan, H.; Fukushima, H.; Taylor, D. M. *J. Polym. Sci., Part A: Polym. Chem.* **1994**, *32*, 1331.
(3) Pinn, E.; Pahler, A.; Saenger, W.; Petsko, G. A.; Green, N. M. *Eur. J. Biochem.* **1982**, *123*, 545.
(4) Bruch, R. C.; White, H. B. *Biochemistry* **1982**, *21*, 5334.
(5) Pugliese, L.; Coda, A.; Malcovati, M.; Bolognesi, M. *J. Mol. Biol.* **1993**, *231*, 698.
(6) Hendrickson, W. A.; Pahler, A.; Smith, J. L.; Sato, Y.; Merrit, E. A.; Phizackerly, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2190.
(7) Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* **1989**, *243*, 85.
(8) Livnah, O.; Bayer, E. D.; Wilchek, M.; Sussman, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5076.

0743-7463/95/2411-3523$09.00/0

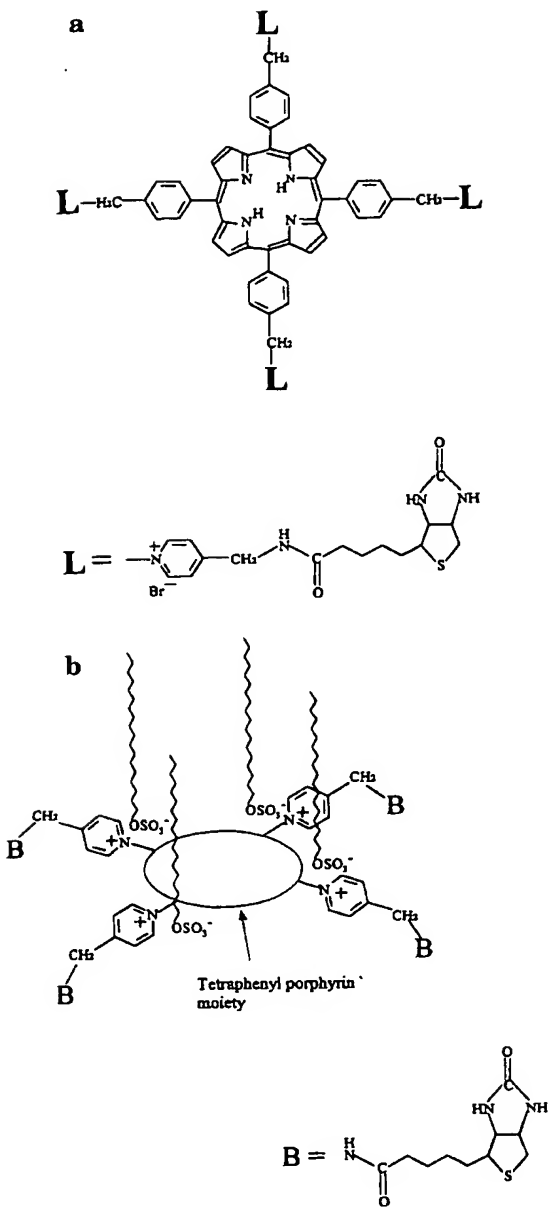


Figure 2. (a) Tetrabiotinylated porphyrin ligand (TBPP) synthesized in this work. (b) TBPP–ODS (1:4) complex which resulted in stable monolayers at the air–water interface.

On the basis of criteria established in our previous study of protein/bisbiotin polymers[2] the above requirements were deemed to be met in the target molecule 5,10,15,20-tetrakis{α-[4-(biotinylamidomethyl)pyridinium bromide]-*p*-tolyl}porphyrin (TBPP) shown in Figure 2a. Porphyrin was chosen as the central molecule owing to the ease with which four biotin moieties may be connected to it. The electrical and optical activity of porphyrin was also an important consideration. The presence of this molecule in the array introduces points of electrical and/or optical stimulation using, for example, techniques based on scanning tunneling microscopy and/or optical near field microscopy.

The strategy adopted for forming the protein–ligand network was based on the successful approach used by Ringsdorf's group for the self-assembly of streptavidin crystals by immobilization to a biotinlipid monolayer at the air–water interface.[9] In our case, this requires the formation of a stable TBPP monolayer at the air–water interface where upon protein injected into the aqueous subphase is allowed to interact with the ligand.

In the following we describe (a) the synthesis of TBPP, (b) the formation of a TBPP–octadecyl sulfate (ODS) complex (Figure 2b) which yields a stable monolayer at the air–water interface, and (c) a preliminary investigation into the immobilization of streptavidin to the TBPP–ODS monolayer.

2. Experimental Section

2.1. Synthetic Strategy. The synthesis of the tetrabiotin ligand was based on the substitution of *p*-bromomethylbenzene units into the 5, 10, 15, and 20 positions in the porphyrin. To impart water solubility to the ligand, pyridinium units in the form of a pyridinium bromide salt were connected to the bromomethyl moieties. Finally, biotin molecules were attached to the *para* position of the pyridene ring to maximize the distances between biotin molecules.

To synthesize the porphyrin skeleton, α-bromo-*p*-tolunitrile was chosen as the starting material and diisobutylaluminium hydride (DIBAL-H) was used to convert the cyano group to aldehyde with 78% yield (Scheme 1). Porphyrin was synthesized by reacting α-bromo-*p*-tolualdehyde (1) and pyrrole with catalytic boron trifluoride etherate (BF_3OEt) in dry $CHCl_3$ following the method of Lindsey et al.[10] After purification using basic alumina column chromatography, 5,10,15,20-tetrakis(α-bromo-*p*-tolyl)-porphyrin (2) was obtained as fine purple colored crystals with 37% yield.

Biotin modification was achieved by introducing the pyridinium group into the carboxylic acid chain. The subsequent reaction between biotinyl-*N*-hydroxysuccinimide (BNHS) and 4-(aminomethyl)pyridine, after silica gel purification, resulted in fine colorless crystals of 4-(biotinylamido)pyridine (3) with 88% yield.

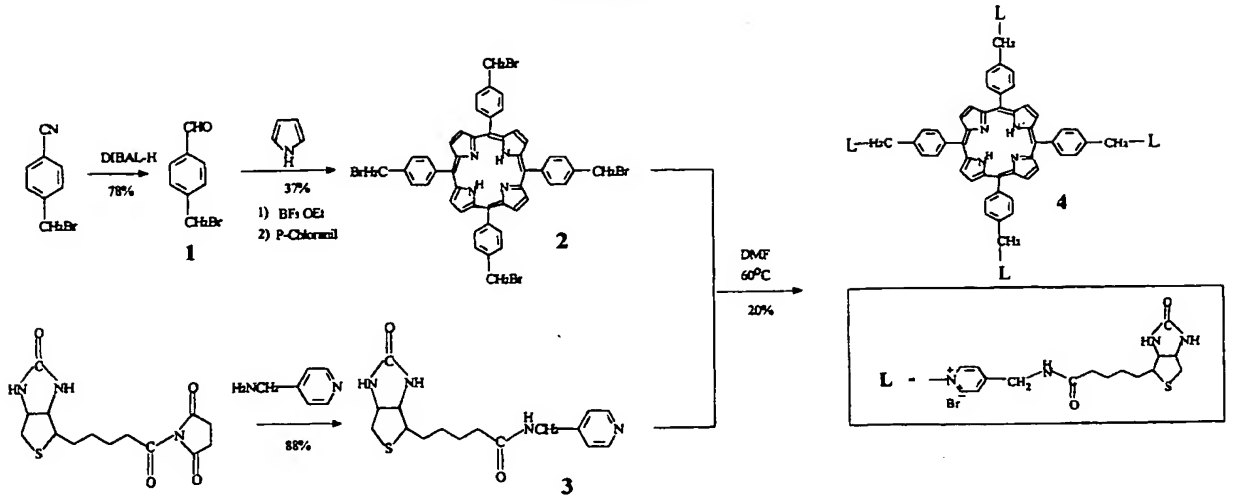
Finally, porphyrin (2) and biotin ester (3) were reacted in dry dimethylformamide (DMF) at 60 °C.[11] Control of reaction temperature at this stage is essential since heating the mixture over 70 °C results in insoluble polymerized substances. Gel filtration using Sephadex G-25 columns was found to be a simple and effective technique for purifying the crude mixture. The tetrabiotin porphyrin (4), obtained as fine purple crystals with 20% yield, gave satisfactory spectroscopic, analytical, and mass spectral data. The Soret band of the biotinylated porphyrin when dissolved in pure water was centered at 415.5 nm.

2.2. Synthesis of TBPP. All reagents, solvents, and chemicals were purchased from Aldrich and Merck and used without further purification. d-(+)-Biotin was purchased from Lancaster Chemicals. DMF, chloroform, and chlorobenzene were stored with molecular sieves prior to use. ^{1}H-NMR spectra of samples were recorded with a Brucker AC-250 (250 MHz) spectrometer and UV–visible spectra obtained with a Hitachi Model U-2000 spectrophotometer. Fast atom bombardment (FAB) analysis was carried out by the SERC Spectrometry Service Centre, University College of Wales, Swansea.

(1) α-Bromo-p-tolualdehyde. A 3.0-g (0.0153 mol) portion of α-bromo-*p*-tolunitrile was dissolved in 50 mL of dry chlorobenzene under nitrogen pressure. The solution was cooled to 0 °C and a 1 M solution of diisobutyl aluminum hydride (DIBAL-H) in 20 mL of hexane was added in a dropwise manner to the solution over 20 min. The mixture was stirred at 0 °C for 1 h and 60 mL of chloroform was added. Subsequently, 10% aqueous HCl was added over 10 min while stirring continued. The organic layer was separated and the aqueous layer was extracted with chlorofrom. This organic extract was then combined with the organic layer and the whole worked up and dried with $MgSO_4$ powder. The chloroform was evaporated and the resultant crystals were recrystallized from a hexane/ethyl acetate (hexane/ethyl acetate 10:1) mixture. The resulting needle crystals were washed with cold hexane to yield 2.38 g (78%) of aldehyde: mp

(9) Blankenburg, R.; Meller, P.; Ringsdorf, H.; Salesse, C. *Biochemistry* **1989**, *28*, 8214.
(10) Lindsey, J. S.; Scheriman, I. C.; Hsu, H. C.; Kearney, P. C.; Maruerettaz, A. M. *J. Org. Chem.* **1987**, *52*, 827.
(11) Milgrom, L. R. *J. Chem. Soc. Perkin Trans. 1* **1983**, 2535.

Scheme 1



98–100 °C; ^{1}H-NMR ($CDCl_3$) δ 10.0 (s, H, –CHO), 7.85 (d, 2H, Ar–H), 7.55 (d, 2H, Ar–H), 4.50 (s, 2H, $-CH_2Br$). Found (C_8H_7OBr): C, 48.54; H, 3.51. Calculated: C, 48.27; H, 3.55%.

(2) 5,10,15,20-Tetrakis(α-bromo-p-tolyl)porphyrin. A 1.0-g (5 mmol) portion of α-bromo-*p*-tolualdehyde (1) and 0.355 g (5 mmol) of pyrrole were dissolved in 400 mL of dry chloroform, and the mixture was stirred at room temperature under nitrogen pressure. A 0.66-mL (1.65 mmol) portion of BF_3Et_2O (2.5 M) in dry chloroform was added to the solution and stirred for 1 h while shielding from ambient light. A 0.942-g (3.75 mmol) portion of tetrachloro-1,4-benzoquinone (TCQ) was added and the mixture was refluxed for 1 h under nitrogen flow. The reaction mixture was filtered to remove the insoluble solid residue. The filtrate was refiltered through Florisil (100–200 mesh; Aldrich) and then evaporated to yield crude crystals. These were washed thoroughly with methanol until the filtrate became colorless and then washed in ether to yield purple crystals. Final purification was carried out using basic alumina column chromatography (solvent, dichloromethane/2% ethyl acetate). The eluted solvent was evaporated and fine purple crystals were obtained (0.45 g, 37%): ^{1}H-NMR ($CDCl_3$) δ 8.75 (s, 8H, pyrrole), 8.10 (d, 8H, Ar-3,5) 7.70 (d, 8H, Ar-2,6) 4.80 (s, 8H, $-CH_2Br$). Found ($C_{48}H_{34}Br_4N_4$): C, 58.64; H, 3.47; N, 5.63. Calculated: C, 58.44; H, 3.47; N, 5.68%. FABMS (*m/z*) 985 (calculated, 986).

(3) 4-(Biotinylamido)Pyridine. A 0.6-g (1.74 mmol) portion of BNHS was dissolved in 20 mL of DMF, and 0.2 g (1.74 mmol) of 4-(aminomethyl)pyridine dissolved in 5 mL of DMF was added. The solution was stirred at 60 °C for 2 h and, subsequently, at room temperature overnight. The DMF solution was removed from the reaction mixture by vacuum and a yellow gelatinous oil was obtained as a residue. The crude mixture was purified by silica gel column chromatography (solvent, chloroform/methanol 8:1). Finally, 0.44 g (75%) of colorless crystals was obtained: mp 195–200 °C; ^{1}H-NMR (DMSO-d_6) δ 8.40 (d, 2H, pyridine-2,6), 8.30 (s, pyridine-CH_2NH–), 7.10 (d, 2H, pyridine-3,5), 6.35 (d, H, $(NH)_2CO$), 6.28 (d, H, $(NH)_2CO$), 4.20 (d, 2H, pyridine-CH_2–), 4.25 and 4.05 (m, 2H, $(CHNH)_2CO$, 3.0 (m, H, –SCH–), 2.70 and 2.72 (dd, 2H, $-SCH_2-$), 2.10 (t, 2H, $-CH_2CONH-$), 1.45 (m, 4H, $-CH_2(CH_2)_2-$), 1.25 (m, 2H, $-CH_2(CH_2)_2-$). Found ($C_{16}H_{22}N_4SO_2$): C, 57.21; H, 6.53; N, 16.46. Calculated: C, 57.46; H, 6.63; N, 16.75%.

(4) 5,10,15,20-Tetrakis{α-[4-(biotinylamidomethyl)pyridinium bromide]-p-tolyl}porphyrin (TBPP). A 0.1-g (0.101 mmol) portion of tetrabromoporphyrin (2) was suspended in 15 mL of dry DMF at 60 °C under nitrogen pressure. Half of 2 was dissolved and the rest was suspended. A 0.15-g (0.445 mmol) portion of pyridinium biotin (3) was dissolved in 6 mL of dry DMF and added dropwise to the partially dissolved porphyrin. The reaction mixture became homogeneous upon adding the solution of 3 whereupon the mixture was stirred at 60 °C under nitrogen flow for 3 h and therefore at room temperature overnight. A large volume of water was added to the reaction mixture and extracted with chloroform. The color of the water layer became red-purple and water with DMF was removed by vacuum evaporation. A red-purple crystalline residue was obtained. A solution of this crude residue in pure water was purified by Sephadex G-15 gel chromatography. The purple colored elution was collected and water removed to obtain 0.03 g (12%) of fine purple crystals: ^{1}H-NMR (DMSO-d_6) 9.35 (d, 8H, pyridine-2,6) 8.80 (s, 8H, pyrrole-H), 8.30 (d, 8H, Ar-3,5), 8.10 (d, 8H, pyridine-3,5), 7.95 (d, 8H, Ar-2,6) 6.8 and 6.7 (d, 8H, $CO(NH)_2$), 6.20 (s, 8H, Ar-CH_2-pyridine), 4.62 (s, 8H, pyridine-CH_2NH–), 4.45 and 4.35 (m, 8H, $(CHNH)_2CO$), 2.90 (m, 4H, –SCH–), 2.75 and 2.60 (dd, 8H, $-SCH_2-$), 2.30 (t, 8H, $-CH_2CONH-$), 1.65 (m, 16H, $-CH_2(CH_2)_2-$), 1.50 (m, 8H, $-CH_2(CH_2)_2-$). Found ($C_{112}H_{112}Br_4N_{20}O_8S_4$): C, 56.87; H, 5.30; N, 11.68. Calculated: C, 57.58; H, 5.62; N, 11.99%.

2.3. Monolayer Studies. *2.3.1. Pressure–Area Isotherms.* The pressure–area (π–A) isotherms were obtained in a sliding barrier, PTFE trough located on an antivibration table in a class 2 semiconductor cleanroom.[12] Pure water was obtained from a Millipore Milli-RO60 reverse osmosis cartridge coupled to a Super Q system comprising ion exchange, organex, and 0.2-μm filter cartridges. The surface pressure was monitored with a Wilhelmy plate and electrobalance to an accuracy of 0.1 mN/m.

For the experiments reported here the subphase was 0.25 M NaCl so as to minimize the nonspecific binding of protein to the TBPP monolayer during protein immobilization studies. The addition of NaCl to the subphase made little difference to the stability of the TBBP monolayer.

The spreading solution was prepared by dissolving 1 mg of TBPP in 1 mL of ultrapure water and then mixing 30 μL of this solution with 0.4 mL of 1:1 methanol/chloroform solution. Pressure–area isotherms were obtained 30 min after spreading an aliquot of the final solution on the subphase surface. The compression rate used was 0.018 (nm^2/molecule)/s.

It was soon apparent that the water solubility of TBPP was too great to allow stable monolayers to form. To prevent loss of material to the subphase, TBPP was complexed with sodium octadecyl sulfate to form a long chain alkanoic salt by replacing Br^- with the octadecyl sulfate (ODS) anion. A similar strategy was adopted by Barraud and co-workers[13–15] who attached four long alkanoic chains as side groups in order to obtain stable monolayers of a tetrapyridinium salt containing porphyrin.

In the present experiments, the complex was formed by mixing 20 μL of sodium octadecyl sulfate (1 g/L in a 2:8 methanol/chloroform mixture) with 30 μL of the aqueous TBPP solution prior to final dilution in the 1:1 methanol/chloroform spreading solvent. The ionic complex formed spontaneously in the resulting solution with a TBPP–ODS mole ratio of 1:4. For other mole

(12) Taylor, D. M.; Oliveira, N. O., Jr.; Morgan, H. *Thin Solid Films* **1989**, *173*, L141.
(13) Ruaudel-Teixier, A.; Barraud, A. *Thin Solid Films* **1983**, *99*, 33.
(14) Palacin, S.; Lesieur, P.; Stefanelli, I.; Barraud, A. *Thin Solid Films* **1988**, *159*, 83.
(15) Parteu, F. I.; Palacin, S.; Ruaudel-Teixier, A.; Barraud, A. *Thin Solid Films* **1992**, *210/211*, 769.

ratios the quantity of ODS added to the TBPP was adjusted accordingly. Isotherms were obtained under the same conditions as described above. Monolayers for UV–vis examination were transferred to a quartz substrate by vertical dipping at a pressure of 30 mN/m and a speed of 4 min/min. Deposition ratios were recorded automatically.

Immobilization of streptavidin (Vector Laboratories, Ltd, Peterborough, U.K.) was carried out using similar procedures to those reported by Blankenburg et al.[9] A microsyringe was used to inject a solution composed of 0.5 mg of protein in 3 mL of 0.25 M NaCl at several positions under a TBPP–ODS monolayer held in the "gaseous" phase ($\pi \sim 0$ mN/m). The monolayer was then left to incubate for 2 h at 29 °C.

2.3.2. Fluorescence Studies. Fluorescence microscopy is a sensitive, nondestructive optical technique for investigating the phase behavior of monolayers at the air–water interface. It has proved particularly useful for investigating the phase transitions of phospholipids[16,17] and has shown that streptavidin immobilized to a biotinlipid monolayer has a domain-like structure.[9] The technique is used here to visualize both the TBPP–ODS monolayer as well as the immobilization of streptavidin. The experimental system consisted of a simple trough for film preparation combined with a fluorescence microscope. Optical excitation of the monolayer was by a 100-W mercury lamp through either blue (400–480 nm) or green (560–595 nm) filters. The fluorescence image was viewed through a filter with low wavelength cut-off-either at 500 nm or at 600 nm using a low-light-level video camera (Hamamatsu C 2400-08-C).

Experiments were performed in which aliquots of the TBPP-ODS (1:4) complex prepared as above were spread on the surface of NaCl subphases ranging from 10 to 250 mM with little effect on the observed images. In the fixed area trough used for the fluorescence measurements, the surface pressure was generally kept close to zero by controlling the amount of complex spread. Microscopic observation of the monolayer began about 30 min after spreading. Fluorescence images of the TBPP–ODS complex were obtained directly, by exciting the Soret bond (415 nm) with blue light or the Q-band (550–600 nm) with green light. In both cases fluorescence occurred in the range 620–750 nm but was much fainter when exciting with green light.

The protein immobilization experiments were carried out by adding fluorescently-labeled streptavidin (SA-5001, Vector Laboratories), dissolved in 1 mL of ultrapure water (1.58×10^{-2} mM), to the subphase. The fluorescent isothiocyanate (FITC) molecules attached to the protein absorbed at ~480 nm and fluoresced at 525 nm. Typically, 10 μL of the stock solution was injected into the subphase at several points and allowed to incubate at 29 °C prior to recording the fluorescence micrographs.

2.3.3. STM Studies. Substrates for monolayer deposition and subsequent STM imaging were prepared by evaporating 500 nm of gold onto freshly cleaved mica held at a temperature of 400 °C. Evaporation was carried out in a turbomolecular system at a pressure of 10^{-6} Torr and at a rate of ~1 nm/s. The gold-coated mica was annealed for a further 12 h at 400 °C under nitrogen to produce atomically flat terraces of gold suitable for STM imaging. (The rms roughness of the gold layers was about 0.03 nm over a 10 nm × 10 nm area). Monolayers of the TBPP–ODS complex were deposited onto the gold films by vertical dipping as above for quartz and also by horizontal lifting at a pressure of 30 mN/m.

Images were obtained using a WA Technology STM (Cambridge, U.K.) with the sample biased at +600 mV with respect to the tip and with a tunneling current of 0.1 nA.

3. Results

The π–A isotherms for TBPP–ODS monolayers obtained with spreading solutions containing different mole ratios of TBPP to ODS are shown in Figure 3. The pure salt was unstable at the air–water surface, tending to early collapse and dissolution in the subphase. Each increase in the mole ratio of ODS in the TBPP–ODS complex increased the stability of the monolayer. The 1:4 TBPP–ODS complex, in particular, was highly stable under compression. For example, after compressing to 30 mN/m, although the area per molecule decreased by about 15% during the first 10 min or so, over the next 3 h the loss of area was negligible. For greater mole fractions of ODS little further change occurred in the isotherms.

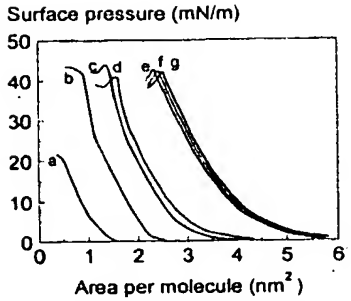


Figure 3. Pressure–area isotherms showing the effect of increasing the mole ratio of ODS in the TBPP–ODS complexes from (a) – to (b) 1, (c) 2, (d) 3, (e) 4, (f) 6, and (g) 10.

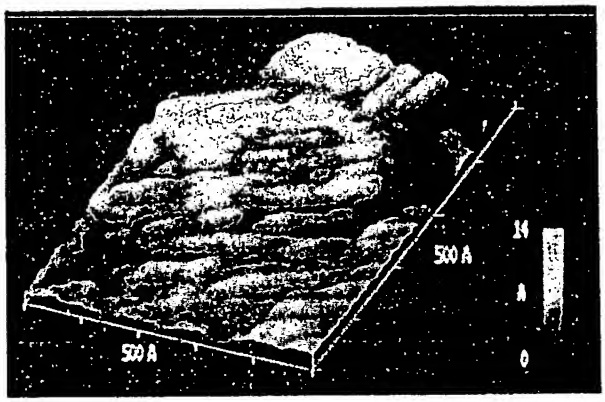


Figure 4. STM image of a monolayer of the TBPP–ODS (1:4) complex deposited onto a gold substrate. The image is 50 nm × 50 nm and resembles an assembly of rodlike stacks.

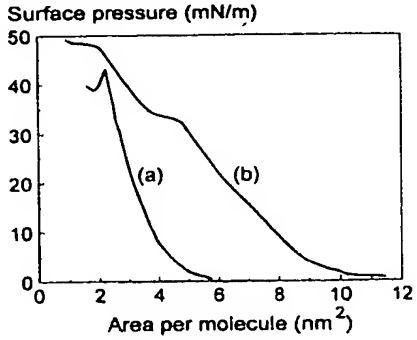


Figure 5. Pressure–area isotherm of (a) the TBPP–ODS (1:4) complex and (b) 2 h after adding active streptavidin to a 0.25 M NaCl subphase at room temperature.

Deposition of the stable monolayer by vertical dipping was readily accomplished albeit with a deposition ratio in the range 1.45–1.64 for the TBPP–ODS (1:4) complex.

An STM image of a monolayer of the TBPP–ODS (1:4) complex deposited onto a gold substrate by horizontal lifting is shown in Figure 4. The size of the image is 50 nm × 50 nm and the height is about 1.2 nm. The monolayer appears to have organized into rodlike stacks with a diameter ~2.8 nm.

Figure 5 shows the effect on the isotherm of the TBPP–ODS (1:4) complex of adding streptavidin to the subphase

(16) Lösche, M.; Möhwald, M. *Rev. Sci. Instrum.* **1984**, *55*, 1968.
(17) Lösche, M.; Helm, C.; Mattes, H. D.; Möhwald, H.; Knoll, W. *Thin Solid Films* **1985**, *133*, 65.

and incubating for 2 h. A clear shift to larger areas has occurred. Addition of inactive protein to the subphase produced no change to the isotherm over the same time period. The same pattern of behavior was observed with avidin. These results confirm that a specific interaction is occurring between protein in the subphase and biotin in the monolayer suggesting in turn that biotin ligands point into, rather than out of, the subphase.

Fluorescence microscopy has been shown to be an invaluable aid to understanding processes that occur at the air–water interface[18] and has been used successfully to image the formation of streptavidin domains below a biotinlipid monolayer.[9,19] Figure 6a is a fluorescence micrograph of a monolayer of the TBPP–ODS (1:4) complex in the low-pressure phase. The "monolayer" appears to be composed of a network of fluorescing porphyrin molecules surrounding dark circular patches of varying size. That the black areas are empty of material was confirmed by adding a second aliquot of the complex to the surface whereupon the fluorescent regions expanded while the darker, circular regions decreased in size.[20] This is opposite to the findings of Möhwald (Figure 3 of ref 18) for L-α-dimyristoylphosphatidic acid (DMPA) where the uniformly-sized, circular domains of condensed material, devoid of fluorescing dye, grew with increasing surface pressure in the gas–fluid coexistence phase.

Figure 6b shows the effect of adding FITC-labeled streptavidin to the subphase and incubating for 2 h. A totally different structure has now formed, with protein seemingly having formed large domain-like features. Eventually, merger of the domains resulted in a uniform fluorescence from the whole of the surface.[20] Similar results were obtained using a sulphorhodamine-labeled streptavidin which enabled the fluorescence from the protein to be distinguished from that of the porphyrin.

4. Discussion

The isotherm for pure TBPP (Figure 3) shows a low collapse pressure (~20 mN/m). The area per molecule was about 1.5 nm^2 at the onset of pressure rise but decreased to less than 0.5 nm^2 per molecule at collapse. The molecular area of tetraphenylporphyrin as determined from the pressure–area isotherm by Bull and Bulkowski[21] is reported to be in the range 0.13–0.17 nm^2. These authors expected an area of 1.6 nm^2 for their flat lying molecule and 0.7 nm^2 if the molecule was vertical. The large discrepancy between experiment and expectation was taken as evidence that, under compression, tetraphenylporphyrin forms multilayer stacks on the water surface.[22] This was confirmed by a smaller than expected d-spacing determined X-ray diffraction from a Y-type film deposited with unity deposition ratio.

Although tilting or stacking of TBPP may occur, in view of the high solubility of the complex (1 mg readily dissolves in 1 mL of water), we believe that diffusion into the subphase is the most likely explanation for the small areas seen here for the pure compound.

Complexation with ODS not only increased the collapse pressure to ~40 mN/m but also shifted the isotherms to larger area. Increasing the mole ratio of ODS in the complex from 0 to 4 increased the molecular area (measured at 20 mN/m) by about 0.7 nm^2 per ODS ion introduced into the complex. The area of a vertically orientated ODS ion is ~0.2 nm^2 and could account for less than a third of the change observed if a simple mixture was being formed. A puzzling feature of the results in Figure 3 is that little change occurred in the isotherm when the mole ratio of ODS increased from 2 to 3. This suggests either that the formation of the 1:3 TMPP–ODS complex is thermodynamically unfavorable or that the presence of the third ODS anion has little effect on the orientation of the porphyrin molecule.

For mole ratios of ODS greater than 4, little further change occurred in the isotherm suggesting that complexation was complete and that any additional ODS simply dissolved in the subphase.

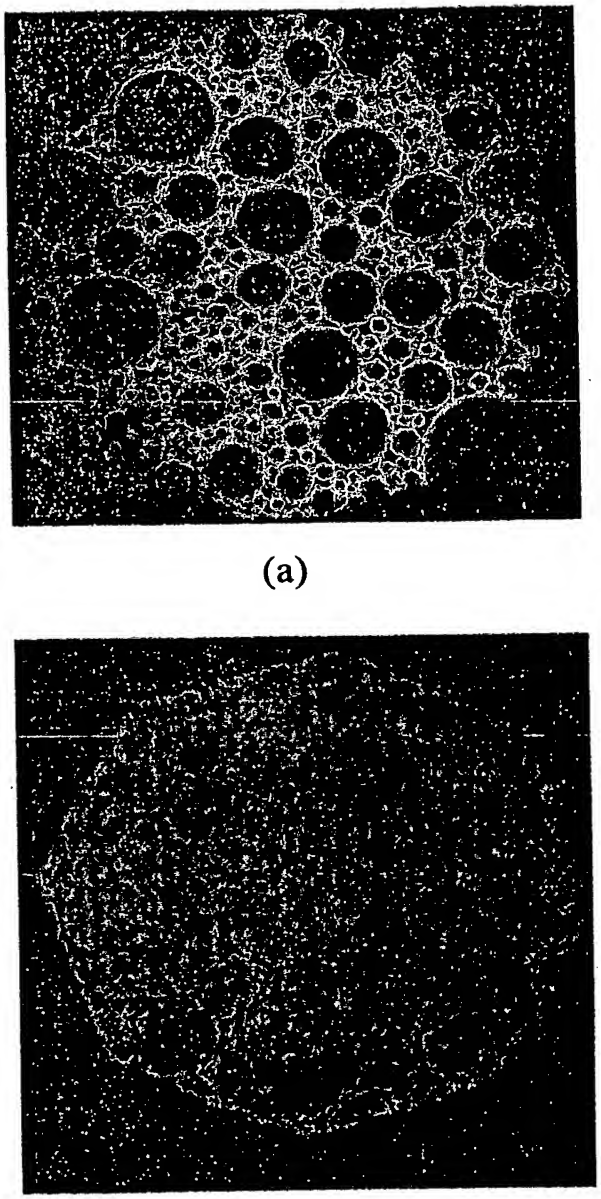


Figure 6. Fluorescence micrographs of a monolayer of the TBPP–ODS (1:4) complex at low surface pressure (a) before and (b) 2 h after adding streptavidin to a 10 mM NaCl subphase at room temperature. In both cases the field of view was 80 μm across.

(18) Möhwald, H. *Thin Solid Films* **1988**, *159*, 1.
(19) Ahlers, M.; Blankenburg, R.; Grainger, D. W.; Meller, P.; Ringsdorf, H.; Salesse, C. *Thin Solid Films* **1989**, *180*, 93.
(20) Fukushima, H.; Taylor, D. M.; Morgan, H.; Ringsdorf, H.; Rump, E. *Thin Solid Films* **1995**, in press.
(21) Bull, R. A.; Bulkowski, J. E. *J. Colloid Interface Sci.* **1983**, *92*, 1.
(22) Hahn, R. A. In *Langmuir-Blodgett Films*; Roberts, G. G., ed.; Plenum Press: New York, 1990; p 59.

The area per molecule of the TBPP–ODS (1:4) complex at 35 mN/m is approximately 2.6 nm^2 significantly greater than the 1.6 nm^2 reported by Ruaudel-Teixier et al.[23] for tetrapyridylporphyrin quaternized with C_{20} alkyl chains at the same surface pressure. These authors estimated the area of their chromophore to be 2.2 nm^2, suggesting that molecules in their monolayer were tilted. On the basis of a simple model of the flat-lying complex which ignores the presence of the biotin ligands, we estimate an area per complex in the range 2.6–3.2 nm^2 suggesting that, for the TBPP–ODS (1:4) complex the porphyrin moiety probably lies flat on the water surface with the biotins pointing into the subphase. The increase in molecular area with increasing mole fraction of ODS is seen, then, as successive steps in the planarization of the porphyrin moiety on the water surface.

The large deposition ratio, ~1.5, indicates that reorganization of the porphyrin molecules must occur during deposition. The STM image in Figure 4 suggests that the molecules are arranged in rodlike stacks, which is certainly feasible given the planar structure of the ligand. Further evidence for this, perhaps, is provided by the red-shift of the Soret band from 415 nm in aqueous solution to 425 nm in the deposited film which may be explained by changes in the $\pi-\pi^*$ excited states of each molecule arising from changes in the degree of overlap of the molecular orbitals.[24]

According to Li et al.[25] the surface coverage of an immobilized porphyrin monolayer may be calculated by applying the Beer–Lambert law to the UV–visible data obtained from the film. Thus, we may write the surface density of molecules, d_s, as $d_s = A\epsilon^{-1}$ where A is the absorbance and ϵ the extinction coefficient. For TBPP in solution ($\lambda_{MAX}(H_2O) = 415$ nm), the extinction coefficient was measured to be $1.93 \times 10^5\ M^{-1}\ cm^{-1}$. Assuming this value applies in the deposited monolayer and noting that the maximum absorbance in the Soret band of the film was 0.064, d_s is estimated to be 3.3×10^{-7} mmol/cm^2, corresponding to ~2 molecules/nm^2. Since both surfaces of the quartz substrate were coated in a monolayer, the area per molecule in the deposited film was ~1 nm^2 which contrasts with ~2.8 nm^2/complex for the same monolayer compressed to 30 mN/m, the deposition pressure (see Figure 3). The discrepancy probably arises from the higher extinction coefficient of the ordered stacks in the film compared with the random orientation in solution.

Addition of either avidin or streptavidin to the subphase below an expanded TBPP–ODS monolayer resulted in the specific adsorption of the protein to the biotinylated complex in exactly the same way as described by Ahlers et al.[19] for immobilization to biotinlipid monolayers. Interestingly, at very low surface pressures, the expansion of the monolayer here is approximately 4.0 nm^2 per complex, some 8 times greater than that observed by Ahlers et al.[19] for biotinylated phospholipid monolayers under similar experimental conditions. This large difference probably reflects the immobilization of protein *within* the monolayer in our case rather than *under* the monolayer in the case of Ahlers et al. In a fully formed 2-D network, since the stoichiometric ratio of protein to tetrabiotinylated porphyrin is 1:1, the expansion should have been ~30 nm^2 per TBPP–ODS complex,[26] almost an order of magnitude greater than that observed. This suggest that after 2 h of incubation, protein has been immobilized to little more than 10% of the monolayer area.

The fluorescence micrographs provide a useful insight into the processes occurring both before and after protein immobilization. In Figure 6a it is seen that, at low surface pressure, the tetrabiotinylated porphyrin forms a 2-D foam on the subphase surface.

A similar foamlike structure is seen in the fluorescence micrographs of streptavidin domains presented by Ahlers et al. (In their Figure 3(a) and (b) in ref 19 protein domains are seen embedded in the foam). They concluded that the dark, circular areas were the "monolayer gas-analogue state". Presumably, the foam was composed of biotinlipid made visible by immobilized protein.

In the present case, when streptavidin molecules are added to the subphase, they attach to biotin moieties and rearrange spontaneously into the domains seen in Figure 6b. The domains, however, are different in shape to those formed under the biotinlipids and evenually merge to form a uniform region of fluorescence.

5. Conclusions

The tetrabiotinylated ligand, 5,10,15,20-tetrakis{α-[4-(biotinylamidomethyl)pyridinium bromide]-*p*-tolyl}-porphyrin (TBPP), has been synthesized. The monolayer behavior of the pure salt, as well complexes formed with different mole fractions of sodium octadecyl sulfate (ODS), has been studied. The TBPP–ODS (1:4) complex formed a highly stable monolayer at the air–water interface. From the pressure–area isotherm, it was deduced that the porphyrin moieties lay flat on the water surface. The monolayer was transferred by vertical dipping onto a solid support but the high deposition ratio (~1.5) suggested that upon transfer, reorganization of the molecules occurred. An STM image of a deposited monolayer of TBPP–ODS (1:4) showed molecules arranged in rodlike stacks.

At low surface pressures, it was shown by fluorescence microscopy that the TBPP–ODS (1:4) complex formed an interconnected network of porphyrin molecules, surrounding circular voids of different size, giving the "monolayer" the appearance of a two-dimensional foam.

Addition of active steptavidin or active avidin to the subphase below an expanded TBPP–ODS monolayer caused an expansion of the pressure–area isotherm. The fluorescence micrographs showed that this was accompanied by a major change in the structure of the monolayer from which it was deduced that the proteins had attached to biotin moieties in the monolayer and spontaneously rearranged into domains. Further work is in progress to determine the structure of these domains.

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(23) Ruaudel-Teixier, A.; Barraud, A.; Belbeoch, B.; Roulliay, M. *Thin Solid Films* **1983**, *99*, 33.
(24) Schicke, G. A.; Scheriman, I. C.; Wagner, R. W.; Lindsey, J. S.; Bocian, D. F. *J. Am. Chem. Soc.* **1989**, *111*, 1344.
(25) Li, D.; Swanson, B. I.; Robinson, J. M.; Hoffbauer, M. A. *J. Am. Chem. Soc.* **1993**, *115*, 6975.
(26) Taylor, D. M.; Morgan, H.; D'Silva, C. *J. Colloid Interface Sci.* **1991**, *144*, 53.

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The specific adsorption of streptavidin to a tetrabiotinylated porphyrin monolayer at the air–water interface

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Abstract

The specific interaction at the air–water interface between streptavidin and a monolayer of the tetrabiotinylated ligand 5,10,15,20-tetrakis {α-[4-(biotinylamidomethyl)pyridinium bromide]-p-tolyl} porphyrin, stabilised by complexation with sodium octadecyl sulphate, is observed directly by surface pressure measurements and by fluorescence microscopy. Changes in the structure of the monolayer, especially the appearance of domains, after adding protein to the subphase confirm that the concomitant expansion of the pressure–area isotherm is caused by the specific adsorption of protein to biotin ligands in the monolayer.

Keywords: Langmuir–Blodgett films; Monolayers; Nanostructures

The assembly of two-dimensional arrays of proteins is beginning to receive considerable interest [1,2] not only from the biological and chemical aspects, e.g. molecular recognition, molecular separation and immunosensing, but also because such arrays could form templates for the assembly of molecular electronic circuits [3]. Ringsdorf and co-workers [4,5] have investigated the two-dimensional crystallisation of streptavidin molecules on biotinlipid monolayers using fluorescence microscopy and have shown that the shape of the protein domains so-formed depend on the detailed structure of the lipid. Recently, Haas and Möhwald [6] using X-ray diffraction techniques have shown that despite the high positional order in such aggregates, the packing density is low because of the presence of associated water molecules. The same reason was suggested by Taylor et al. [7] to explain the greater molecular area estimated for avidin from pressure–area isotherms compared with X-ray crystallographic studies [8].

In the present communication, we report on the surface behaviour of a tetrabiotinylated porphyrin ligand which was specially synthesised in an attempt to form an interlinked two-dimensional protein array at the air–water interface, shown in idealised form in Fig. 1. This approach to network formation was based on the well-known high affinity of streptavidin for its complementary ligand, biotin. The binding

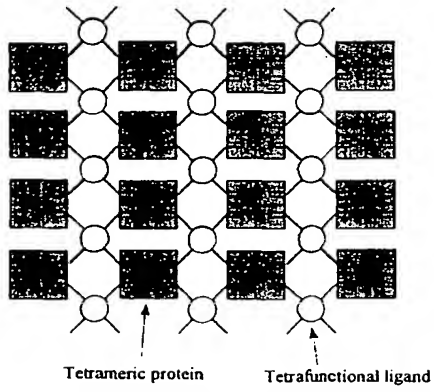

Fig. 1. An ideal two-dimensional protein array in which a tetrafunctionalised ligand is used to bind tetrameric proteins.

constant, $\sim 10^{15}$ M^{-1}, leads to a protein–ligand interaction whose strength is matched only by systems involving liganded metal ions either as partial covalent bonds or chelates. Steptavidin, a highly stable, robust protein which binds biotin almost irreversibly over a wide range of pH at room temperature, is, therefore, an ideal candidate for the formation of molecular networks.

The ligand 5,10,15,20-tetrakis {α-[4-(biotinylamidomethyl)pyridinium bromide]-p-tolyl} porphyrin (TBPP) is shown in Fig. 2. Details of the synthetic strategy have been given elsewhere [9]. Briefly, it was based on the substitution

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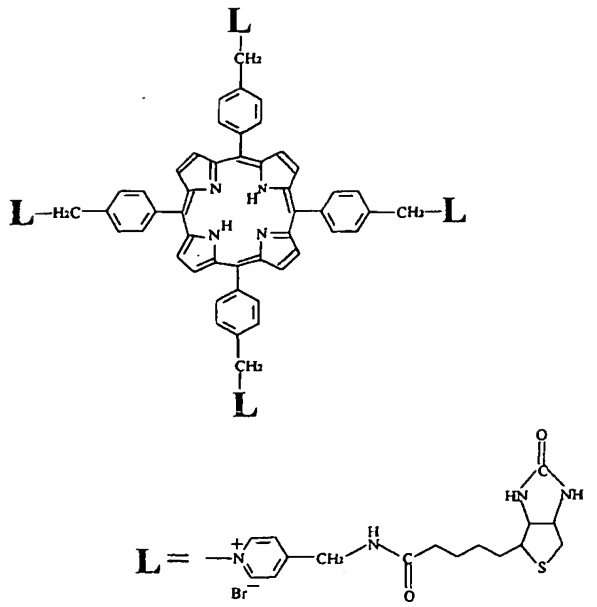

Fig. 2. The tetrabiotinylated ligand TBPP synthesised in the present work.

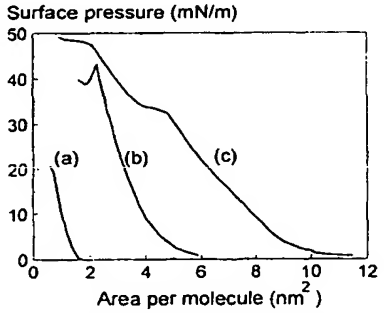

Fig. 3. Pressure–area isotherms for (a) pure TBPP and for the TBPP-ODS (1:4) complex (b) before and (c) after addition of streptavidin to the subphase ($T = 20$ °C).

of p-bromo-methyl benzene units into the 5, 10, 15 and 20 positions in the porphyrin, to which pyridinium units in the form of a pyridinium bromide salt were connected. Finally, biotin molecules were attached to the para position of the pyridene ring to maximise the distance between biotin moieties. A solution of the crude product in water was purified by Sephadex G-15 gel chromatography. Evaporation of water from the elutant yielded the final product as fine purple crystals which showed excellent elemental analysis (Found ($C_{112}H_{112}Br_4N_{20}O_8S_4$): C (56.87%), H (5.30%), N (11.68%); Calculated: C (57.58%), H (5.62%), N (11.99%)) confirmed by ^{1}H NMR and fast atom bombardment analysis.

Spreading solutions of TBBP were prepared by dissolving 1 mg of the compound in 1 ml of ultrapure water then mixing 30 μl of this stock solution with 0.4 ml of a 1:1 methanol/chloroform solution. When spread at the air–water interface in a sliding-barrier Langmuir trough [10] monolayers of pure TBPP were unstable, displaying both a low collapse pressure (~ 20 mN m^{-1}) and low areas per complex (< 0.5 nm^2) as can be seen in Fig. 3. While this may have been caused in part by tilting and stacking of the porphyrin units as suggested for tetraphenylporphyrin [11], we believe that the main reason here is the high water solubility of the TBPP salt.

Stable monolayers could only be formed by complexing TBPP with sodium octadecyl sulphate (Fig. 4). This was achieved by mixing 20 μl of sodium octadecyl sulphate (ODS) (1 g l^{-1} in a 2:8 methanol/chloroform mixture) with 30 μl of the aqueous TBBP solution prior to final dilution in the 1:1 methanol/chloroform spreading solution. By replacing the Br^- with the long-chain octadecyl sulphate (ODS) anion sufficient hydrophobicity was imparted to TBPP to overcome its water solubility, hence allowing stable isotherms to be obtained.

Fig. 3(b) is the pressure–area isotherm obtained at room temperature upon spreading the TBPP-ODS complex in which the mole ratio was 1:4. While lower mole ratios of ODS also result in stable isotherms, the fully saturated complex (Fig. 4) is the most stable and the most expanded. When compressed to 30 mN m^{-1} the area of the 1:4 complex, while decreasing by about 20% in the first 10–15 min, thereafter decreased by only 3% in the next 3 h.

The collapse pressure rose to ~ 40 mN m^{-1} after complexation with ODS and the area per complex at 35 mN m^{-1} was ~ 2.6 nm^2, close to our estimate of 2.6 to 3.2 nm^2 based on a crude molecular model in which the tetrapyridyltetraphenylporphyrin ligand is assumed to lie flat on the water surface with the biotin moieties pointing down into the subphase. (Ruaudel-Teixier and Barraud [12] suggest an area per molecule of 2.2 nm^2 for a tetrapyridylporphyrin quaternised with C_{20} alkyl chains).

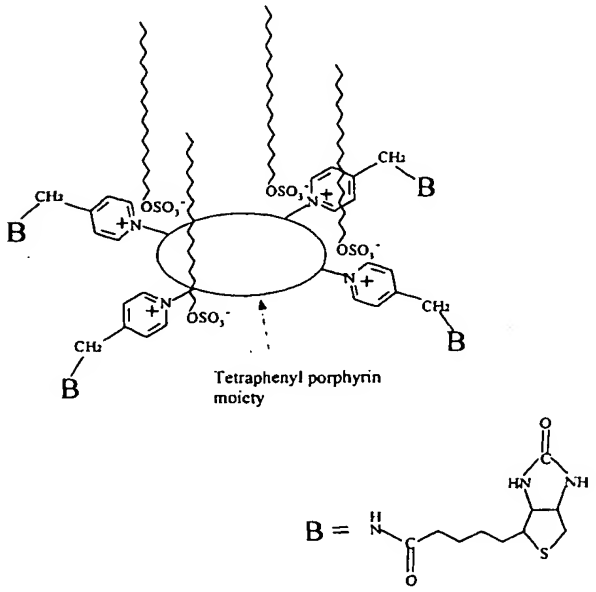

Fig. 4. The TBPP-ODS (1:4) complex used in the protein immobilisation experiments.

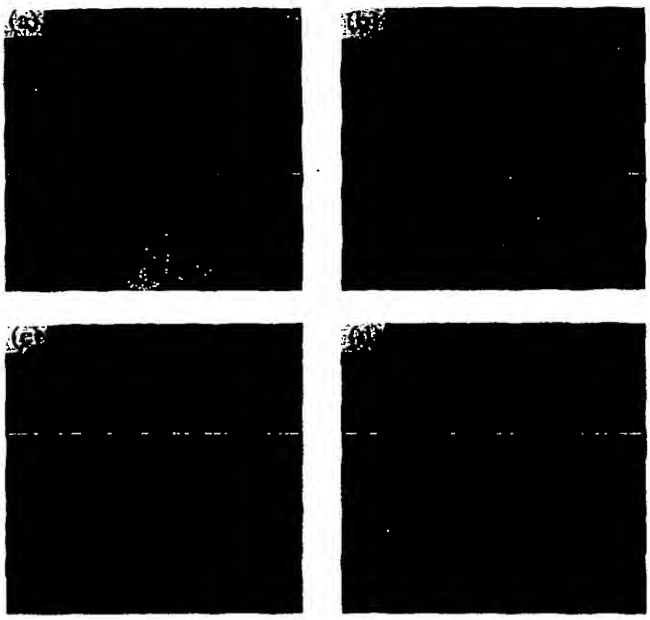

Fig. 5. Fluorescence micrographs of (a) an expanded monolayer of the TBPP-ODS (1:4) complex, (b) after addition of a second aliquot of the complex, (c) 2 h and (d) 3 h after injecting streptavidin into the subphase under an expanded monolayer such as that in (a). In all cases the field of view is 80 mm across. $T = 20$ °C.

Addition of 10 μl of streptavidin (Vector Laboratories Ltd, Peterborough) in ultrapure water (10 mg ml^{-1}) to the subphase (~1 l of 0.25 M NaCl to minimise non-specific electrostatic interaction) just below an expanded monolayer of the TBPP-ODS (1:4) complex and incubating for 2 h at 29 °C produced a further expansion of the monolayer (Fig. 3). At low surface pressure, the expansion of the monolayer was approximately 3.0 nm^2 per complex, some six times greater than that observed by Ahlers et al. [5] for adsorption to a biotinlipid monolayer. In a fully formed two-dimensional network (Fig. 1) the mole ratio of protein to tetrabiotinylated ligand should be 1:1, from which an expansion > 30 nm^2 per TBPP-ODS complex was expected [7]. Thus after 2 h of incubation, protein had apparently adsorbed to only ~10% of the monolayer area. This may be because of the poor distribution and low diffusion rates of the protein in the subphase. Addition of streptavidin, previously inactivated by mixing with excess biotin, had little effect on the isotherm of TBPP-ODS [8] confirming the specific nature of the streptavidin/biotin interaction in Fig. 3.

Fig. 5(a) is a fluorescence micrograph of an expanded monolayer of the TBPP-ODS (1:4) complex. The image was obtained by optical excitation with a 100 W mercury lamp through a 400–480 nm band pass filter so as to excite the Soret band of the porphyrin (~415 nm). The resulting fluorescence, viewed through a filter with a low wavelength cut-off of ~600 nm, was detected by a low-light-level camera (Hamamatsu C 2400-08-C). The monolayer is seen to be composed of a network of fluorescing porphyrin moieties surrounding dark, circular patches of varying size. That the circular patches are devoid of material was confirmed by holding the area fixed and spreading a second aliquot of the complex on the surface whereupon the areas of fluorescence expanded but the circular patches contracted (Fig. 5(b)).

The effect of adding FITC-labelled streptavidin (SA-5001, Vector Laboratories Ltd) to the subphase was dramatic. After 2 h of incubation at 29 °C, the fluorescence micrograph in Fig. 5(c) was obtained by viewing through a filter with a low wavelength cut-off of ~500 nm. It is seen that the original network structure had been replaced by a domain-like structure. After 3 h, these domains appear to fuse together to yield an almost uniform fluorescence from the surface (Fig. 5(d)). When inactive streptavidin was injected into the subphase, the foam-like structure in Fig. 5(a) remained unchanged, confirming the specific nature of the streptavidin/biotin interaction in Fig. 5(c) and 5(d).

In order to distinguish the contributions of the protein from the porphyrin in the above images a separate experiment was carried out in which sulphorhodamine-labelled streptavidin was injected into the subphase. In this case the monolayer was excited by light at ~550 nm and the emission observed through a narrow pass band filter with a centre wavelength of 625 nm. Little fluorescence from the rhodamine-labelled streptavidin was seen in the circular regions devoid of porphyrin. As incubation proceeded identical behaviour to that in Fig. 5(a) and 5(c) was observed again confirming the specific nature of the interaction.

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References

[1] K. Nagayama, 1994, in H. Masura et al. (eds.), *Microchemistry: Proc. JRCD/KUL Int. Symp. on Spectroscopy and Chemistry in Small Domains*, North-Holland, Amsterdam, p. 173.
[2] H. Yoshimura, T. Scheybani, W. Baumeister and K. Nagayama, *Langmuir, 10* (1994) 3290.
[3] H. Morgan, H. Fukushima and D.M. Taylor, *J. Polym. Sci. Part A: Polym. Chem., 32* (1994) 1331.
[4] R. Blankenburg, P. Meller, H. Ringsdorf and C. Salesse, *Biochemistry, 28* (1989) 8214.
[5] M. Ahlers, R. Blankenburg, D.W. Grainger, P. Meller, H. Ringsdorf and C. Salesse, *Thin Solid Films, 180* (1989) 93.
[6] H. Haas and H. Möhwald, *Langmuir, 10* (1994) 363.
[7] D.M. Taylor, H. Morgan and C. D'Silva, *J. Colloid Interface Sci., 144* (1991) 53.
[8] N.M. Green and M.A. Joynson, *Biochem. J., 118* (1970) 71.
[9] H. Fukushima, H. Morgan and D.M. Taylor, *Thin Solid Films, 244* (1994) 789.
[10] D.M. Taylor, O.N. Oliveira, Jr., and H. Morgan, *Thin Solid Films, 173* (1989) L141.
[11] R.A. Hahn, in G.G. Roberts (ed.), *Langmuir–Blodgett Films*, Plenum Press, New York, 1990, p. 59.
[12] A. Ruaudel-Teixier and A. Barraud, *Thin Solid Films, 99* (1983) 33.

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Notes & Tips

Synthesis of biotinylated heme and its application to panning heme-binding proteins

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Iron-protoporphyrin IX, called "protoheme" or simply "heme," plays a variety of roles as the active center in many proteins such as enzymes, oxygen carriers, and biological sensors [1]. Thus detection and separation of heme-binding proteins are important for investigating these physiological functions. Also, in recent years, design and laboratory evolution of hemoproteins, in which selection of functional clones is an essential step, has been a major subject in the study of structure–function relationships [2–5]. Previously, hemin-agarose was synthesized and used for affinity chromatography of hemoproteins [6]. However, the interference of agarose beads with spectroscopic measurements makes it difficult to detect the specific ligation of proteins to the heme. Furthermore, nonspecific interactions between the proteins and the agarose resin allow the experimental results to be ambiguous. Here, to avoid these problems, we have prepared biotinylated heme (Fig. 1) and investigated its usage for detection and purification of hemoproteins. Biotin is widely utilized for affinity selection, labeling, and isolation of proteins, DNA, carbohydrates, membranes, and cells, through its tight and specific binding to streptavidin, and a variety of streptavidin derivatives have been synthesized and are commercially available [7,8]. The purification of native and artificial heme-binding proteins from recombinant cell extracts using biotinyl heme is demonstrated.

Synthesis and purification of biotinyl heme

Iron-protoporphyrin IX chloride (hemin) and *N*-[5-(hydrazinocarboxy)pentyl]-D-biotinamide (biotin hydrazide) were dissolved in anhydrous DMF[1] and DMSO at 4.4 and 50 mg/ml, respectively. Twenty microliters of the biotin hydrazide solution and 5.6 mg of DCC were added to 1 ml of the hemin solution. The reaction mixture was gently shaken and incubated in the dark for 3 h at room temperature. To conjugate only one of the two propionate groups of protoheme with biotin hydrazide, approximately 2.5 equivalent excess amoünts of hemin were used for the reaction. Hemin and biotin hydrazide were purchased from Sigma and Vector Laboratories, respectively.

The reaction mixture prepared as above was supplemented with approximately 5% (v/v) pyridine and was applied onto a C18 reverse-phase preparative HPLC column, COSMOSIL 5C18-ARII (Nacalai Tesque). The biotinyl heme was eluted with a gradient of 40–60% acetonitrile in the presence of 0.1% TFA. The peak fraction containing the biotinyl heme was collected and immediately lyophilized in the dark. The sample was dissolved in a minimal volume of DMSO and stored at −80 °C. Analytical HPLC revealed that the purity of the sample was more than 95%. The correct identity of the purified molecule was verified by MALDI/TOFMS with a Reflex mass spectrometer (Bruker Daltonik, Germany) using 2,5-dihydroxybenzoic acid as the matrix in the reflectron positive mode. The molecule had a mass of 969.4 Da, which corresponds to the calculated mass of

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[1] *Abbreviations used:* DCC, dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethyl formamide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; OGP, 1-*o*-*n*-octyl-β-D-glucopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; TOFMS, time-of-flight mass spectrometry; UV–Vis, ultraviolet–visible light.

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doi:10.1016/S0003-2697(03)00423-8

redox variants: evolutionary strategies for modulating redox potential revealed using a library approach, Biochemistry 41 (2002) 4321–4328.
[3] N.R. Rojas, S. Kamtekar, C.T. Simons, J.E. McLean, K.M. Vogel, T.G. Spiro, R.S. Farid, M.H. Hecht, De novo heme proteins from designed combinatorial libraries, Protein Sci. 6 (1997) 2512–2524.
[4] B.R. Gibney, F. Rabanal, J.J. Skalicky, A.J. Wand, P.L. Dutton, Iterative protein redesign, J. Am. Chem. Soc. 121 (1999) 4952–4960.
[5] Y. Isogai, A. Ishii, T. Fujisawa, M. Ota, K. Nishikawa, Redesign of artificial globins: effects of residue replacements at hydrophobic sites on the structural properties, Biochemistry 39 (2000) 5683–5690.
[6] K. Tsutsui, G.C. Mueller, Affinity chromatography of heme-binding proteins: an improved method for the synthesis of hemin-agarose, Anal. Biochem. 121 (1982) 244–250.
[7] H. Heitzmann, F.M. Richards, Use of the avidin–biotin complex for specific staining of biological membranes in electron microscopy, Proc. Natl. Acad. Sci. USA 71 (1974) 3537–3541.
[8] E.A. Bayer, M. Wilchek, The use of the avidin-biotin complex as a tool in molecular biology, Methods Biochem. Anal. 26 (1980) 1–45.
[9] F. Ascoli, M.R. Fanelli, E. Antonini, Preparation and properties of apohemoglobin and reconstituted hemoglobins, Methods Enzymol. 76 (1981) 72–87.
[10] E. Antonini, M. Brunori, Hemoglobin and myoglobin in their reactions with ligands, Frontiers of Biology, vol. 21, North-Holland, Amsterdam, London, 1971.
[11] B.A. Springer, S.G. Sligar, High-level expression of sperm whale myoglobin in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 84 (1987) 8961–8965.
[12] Y. Isogai, M. Ota, T. Fujisawa, H. Izuno, M. Mukai, H. Nakamura, T. Iizuka, K. Nishikawa, Design and synthesis of a globin fold, Biochemistry 38 (1999) 7431–7443.
[13] B.R. Gibney, F. Rabanal, K.S. Reddy, P.L. Dutton, Effect of four helix bundle topology on heme binding and redox properties, Biochemistry 37 (1998) 4635–4643.
[14] H. Schagger, G. Von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal Biochem. 166 (1987) 368–379.

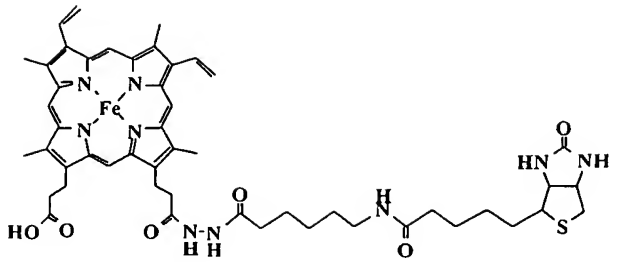

Fig. 1. Structure of biotinyl heme. Protoheme was biotinylated by conjugating one of the two propionate groups of the heme to biotin hydrazide with the 6-aminohexanoate spacer in carbodiimide coupling.

the biotinyl heme (969.98 Da) in which one of the two propionate groups of protoheme was conjugated with biotin hydrazide (see Fig. 1); 0.6 mg of the biotinyl heme was obtained from the reaction mixture. The yields of the purified molecule were 21 and 8.5% for biotin hydrazide and hemin, respectively.

Reconstitution of myoglobin with biotinyl heme

Apomyoglobin was prepared from horse heart metmyoglobin using the methyl ethyl ketone extraction method described by Ascoli et al. [9]. The heme-removed apoprotein was dialyzed against TN buffer containing 10 mM Tris–HCl (pH 8.0) and 200 mM NaCl at 4 °C. After removal of the insoluble fractions by centrifugation, the supernatant was concentrated to 1–2 mM using Centriprep-3 (Amicon). Reconstitution of myoglobin with the biotinyl heme was performed by addition of the biotin-heme solution containing 20% pyridine into the apomyoglobin solution in increments of 0.1–0.2 equivalents to a small excess of the protein. This mixture was incubated for more than 30 min at 4 °C and was centrifuged at 20,000*g* for 30 min. The reconstituted myoglobin was collected in the supernatant and maintained significant stability similar to that of natural metmyoglobin as judged by measurements of the UV–Vis absorption spectrum (see below). Residual DMSO and pyridine was removed from the heme-protein solution using a Sephadex-G25 desalting column, PD-10 (Amersham Biosciences).

The reconstituted biotin-heme myoglobin exhibited an absorption spectrum identical to that of metmyoglobin as shown in Fig. 2A. The ferric form was reduced into the ferrous deoxy form by addition of $Na_2S_2O_4$ under anaerobic conditions (solid line in Fig. 2B). Weak flow of air or carbon monoxide gas to the solution converted it to the ferrous oxy or CO-bound forms (dotted and broken lines in Fig. 2B). These absorption spectra are indistinguishable from those of native myoglobins [10]. It is noticeable that the biotin-heme myoglobin maintained stable O_2-binding ability or the

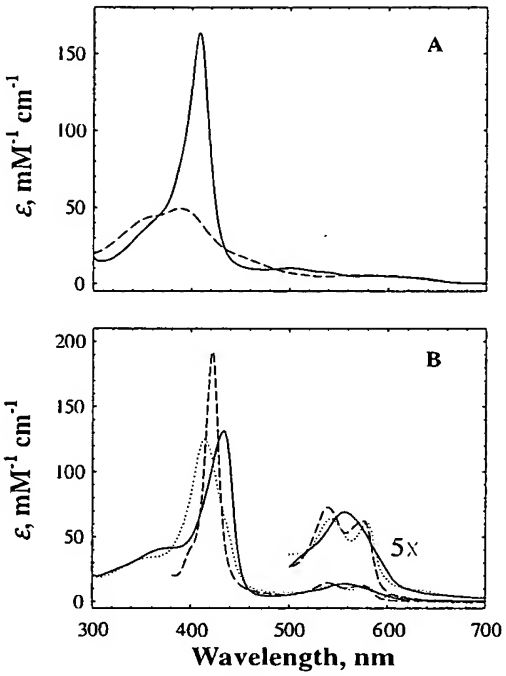

Fig. 2. Absorption spectra of myoglobin reconstituted with the biotinyl heme. The reconstituted myoglobin with the biotinyl heme was diluted with TN buffer to 10–20 μM and the absorption spectra were recorded with a Hitachi U-3000 spectrophotometer using a quartz cuvette of 1.0 cm in path length. Solid and broken lines in A indicate the spectra of the ferric forms of the reconstituted myoglobin and the free biotinyl heme, respectively. Solid, dotted, and broken lines in B indicate the spectra of ferrous deoxy, ferrous oxy, and ferrous CO-bound forms. These spectra of myoglobin with the biotinyl heme are indistinguishable from those of the authentic myoglobins. The ferrous deoxy, oxy, and CO forms were prepared from the ferric form for the spectroscopic measurements according to [10].

biological function. These results suggest that the biotinyl heme is incorporated in the heme pocket of myoglobin in a manner similar to that of normal protoheme in myoglobin.

Purification of recombinant apohemoproteins from cell extracts

Synthetic genes encoding sperm whale myoglobin [11], designed globin-1 (DG1) [12], and designed four-helix-bundle hemoprotein (dA1) were cloned into a pRSET-C vector (Invitrogen). The amino acid sequence of dA1, ML·KKLREEA·LKLLEEF·KKLLEEH·LKWLEGGGGGGGGELLKL·HEELLKK·FEELLKL·AEERLKK·L, was designed to form a four-helix bundle in the dimer and to bind one heme per monomer via bis-His ligation between the two helices [13]. These hemoprotein-coding vectors were transformed into *Escherichia coli* strain BL21 (DE3) and expressed in Terrific Broth medium supplemented with 100 mg/L

ampicillin under the control of T7 promoter using IPTG. Cells were harvested by centrifugation and were washed with 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA. The resultant pellets were suspended in a lysis buffer containing 6 M urea, 0.5 M NaCl, 1 mM EDTA, and 0.1% OGP and were lysed by sonication. After removal of the insoluble fractions by centrifugation, the supernatants were collected and dialyzed against TN buffer. During these procedures, almost all the heme associated with proteins in the cell extracts was removed and the proteins were refolded. After removal of the insoluble fractions by centrifugation, the proteins were concentrated to an appropriate concentration using Centriprep-3 (Amicon). The cell extracts obtained as above were used as the starting materials, from which the recombinant apohemoproteins were purified using the biotinyl heme.

The biotinyl heme was added to the cell extracts prepared as above in small increments to 10–40 μM and was incubated at 4 °C for more than 30 min. The extracts (0.2 ml) obtained from 10 ml of the cultures, which contained the recombinant apohemoproteins at 20–60 μM were used. The addition of the biotinyl heme into the cell extracts induced the intense Soret absorbance bands characteristic of the bound heme in these proteins (not shown), indicating that it was effectively incorporated into the proteins even in the dense mixtures of biological molecules. After removal of the insoluble materials by centrifugation, the solutions were transferred into a sample tube containing streptavidin magnetic beads (MagnaBind streptavidin beads, Pierce) prewashed with a washing buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 0.5% (v/v) Tween 20. The resultant protein–biotin–heme–streptavidin complexes were collected using a magnet. The pellets were washed five-times with the washing buffer and incubated with 10 M imidazole (pH 8.0) to elute the bound proteins. After the removal of the magnetic beads, the solutions were desalted and lyophilized. The lyophilized samples were dissolved in a small amount of TN buffer and analyzed by SDS-PAGE (Fig. 3). The apohemoproteins were purified without significant contamination by other proteins, with 5–10% recovery against the original protein contents in the cell extracts.

The proteins were also eluted by the addition of either acids or denaturants such as guanidine hydrochloride. However, in these cases, denatured streptavidin subunits that are not conjugated to the beads and biotinyl heme were coeluted with the heme proteins. The heme proteins were also purified using streptavidin agarose for the magnetic beads. However, the use of the agarose increased contamination due to nonspecific interactions of proteins with agarose (data not shown).

In the present study, heme was biotinylated by conjugating a propionate group of the heme to biotin hydrazide. The biotinyl heme was efficiently incorporated into native and artificial apohemoproteins and can be recovered in the form of a reconstituted heme protein with a variety of biochemical and immunochemical methods using streptavidin derivatives. In contrast to hemin-agarose, the biotinyl heme is saved from nonspecific interactions of proteins with the agarose resin. Furthermore, the specific binding with hemoproteins can be spectroscopically monitored. Thus the biotinyl heme is useful for detection, purification, and panning of heme-binding proteins from biological materials.

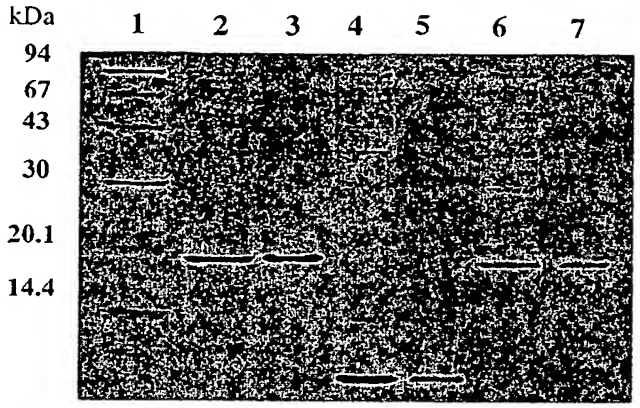

Fig. 3. SDS–PAGE profiles showing the purification of recombinant hemoproteins by the biotinyl heme. Lanes 2 and 3 are the cell extract and the purified fraction of myoglobin, respectively. Lanes 4 and 5 are the cell extract and the purified fraction of designed four-helix bundle hemoprotein (dA1), respectively. Lanes 6 and 7 are the cell extract and the purified fraction of designed globin-1 (DG1), respectively. Lane 1 is the molecular size marker: phosphorylase *b* (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The electrophoresis was carried out with 15% (w/v) polyacrylamide gel [14].

Acknowledgments

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References

[1] A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt (Eds.), Handbook of Metalloproteins, vol. 1, Wiley, New York, 2001.
[2] S.L. Springs, S.E. Bass, G. Bowman, I. Nodelman, C.E. Schutt, G.L. McLendon, A multigeneration analysis of cytochrome b_{562}